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(54) Title:　HUMAN LEUKOCYTE 12-LIPOXYGENASE AND ITS ROLE IN THE PATHOGENESIS OF DISEASE STATES

(57) Abstract

Evidence for the presence of a human leukocyte type of 12-LO enzyme and its role in the pathogenesis of several major disease states or processes, including atherosclerosis, breast cancer, autoimmune and inflammatory disease, diabetic vascular and kidney disease and insulin resistance is described. Mediation, including the blockage of the human leukocyte 12-LO expression or enzyme activation, provides novel treatment to prevent or ameliorate these disease states.

HUMAN LEUKOCYTE 12-LIPOXYGENASE AND ITS ROLE IN THE PATHOGENESIS OF DISEASE STATES

RELATED APPLICATIONS

This application is a continuation-in-part of U.S.
5 patent application Serial No. 08/434,681, filed 5 May
1995, which is a continuation-in-part of application
PCT/US94/00089, filed 4 January 1994, which is a
continuation-in-part of U.S. patent application Serial
No. 07/936,660, filed 28 August 1992.

10 ## GOVERNMENT RIGHTS STATEMENT

This invention was made with government support
under Grant No. DK 39721 RO1 awarded by the National
Institutes of Health. The government has certain
rights in the invention.

15 ## TECHNICAL FIELD

This invention pertains to a human leucocyte type
of 12-LO and its role in the pathogenesis of several
major disease states.

ABBREVIATIONS

20 AA   = Arachidonic acid
AII   = Angiotensin II
EGF   = Epidermal Growth Factor
FN   = Fibronectin
GAPDH   = Glyceraldehyde-3-phosphate
25   dehydrogenase
GF   = Growth Factor
HAEC   = Human Aortic Endothelial Cells
HETE   = Hydroxyeicosatetraenoic Acid
12 HETE   = 12-Hydroxyeicosatetraenoic Acid
30 HG   = High Glucose

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	hl 12-LO	=	Human Leukocyte 12-Lipoxygenase
	hl 15-LO	=	Human Leukocyte 15-Lipoxygenase
	HODE	=	Hydroxyoctadecadienoic acid
	12-HPETE	=	12-Hydroperoxyeicosatetraenoic Acid
5	HSMC	=	Human Aortic Smooth Muscle Cells
	HPLC	=	High Pressure Liquid Chromatography
	IL-1	=	Interleukin-1
	LDL	=	Low Density Lipoprotein
	LO	=	Lipoxygenase
10	12-LO	=	12-Lipoxygenase
	15-LO	=	15-Lipoxygenase
	MAPK	=	Mitogen Activated Kinase
	mmLDL	=	Minimally Modified Low Density Lipoprotein
15	MO	=	Monocytes
	NIDDM	=	Non-insulin Dependent Diabetes Mellitus
	NG	=	Normal Glucose
	PDGF	=	Platelet Derived Growth Factor
	PKC	=	Protein Kinase C
20	pl 12-LO	=	Human platelet 12-Lipoxygenase
	PVSMC	=	Porcine Vascular Smooth Muscle Cells
	RT-PCR	=	Reverse Transcriptase Polymerase Chain Reaction
	SMC	=	Smooth Muscle Cells
25	TGFB	=	Transforming Growth Factor Beta
	TNF	=	Tumor Necrosis Factor 1
	VSMC	=	Vascular Smooth Muscle Cells

BACKGROUND OF THE INVENTION

The three mammalian lipoxygenases are named according to the carbon position (1, 2 or 3) at which they oxygenate arachidonic acid (4). There is increasing evidence that certain LO enzymes are involved in the pathogenesis and acceleration of atherosclerosis by inducing oxidation of LDL to its atherogenic form (5,6) and increasing the growth or migration of smooth muscle cells (1, 7-9). In addition, evidence suggests that a 12-LO protein plays a role in mediating angiotensin II (AII) induced vascular and adrenal actions (10-12). Recent studies indicate that at least two forms of 12-LO exist, i.e., pl 12-LO cloned from human erythroleukemia cells (2,13) and a porcine leukocyte 12-LO which has been isolated and cloned from porcine mononuclear cells, pituitary (14) and bovine tracheal cells (15).

Applicants have demonstrated the presence of a leukocyte type of 12-LO in human adrenal glomerulose cells (3). The human 15-LO has been purified from human and rabbit reticulocytes (16,17). The human platelet and porcine leukocyte type 12-LO share 65% amino acid homology (13). However, porcine leukocyte type 12-LO is highly homologous to human 15-LO (86%) (14). Recently, it has been shown that 15-LO is expressed in macrophages of human atherosclerotic lesions but not in unstimulated monocytes (18).

SUMMARY OF THE INVENTION

This application describes evidence for the presence of a human leucocyte type of 12-LO enzyme (hl 12-LO) and its role in the pathogenesis of several major disease states or processes, including atherosclerosis, breast cancer, autoimmune and inflammatory disease, diabetic vascular and kidney

disease and insulin resistance. There are several
features of this unique enzyme that can link several
seemingly diverse conditions.

     1.   hl 12-LO can utilize arachidonic and linoleic
acid as fatty acid substrates generating hydroperoxides
and other lipid mediators which can activate important
signal transduction pathways commonly associated with
these disorders. These mediators include (a) kinases
such as specific isoforms of protein kinase C and
mitogen activated kineses (MAPK), (b) transcription
factors such as NFkB and oncogenes which have clearly
been implicated in inflammatory and autoimmune
conditions, atherosclerosis, cancer growth and
metastasis.

     2.   Activation of the hl 12-LO enzyme can itself
generate superoxide anions which can lead to the
propagation of free radical processes which could
accelerate the oxidative modification of lipids and
proteins. These processes are involved in the
pathogenesis of inflammatory, neoplastic and
atherogenic conditions.

     3.   The hl 12-LO enzyme is strategically located.
Evidence is presented showing the presence of the
leucocyte type 12-LO in human monocytes, aortic
vascular smooth muscle and endothelial cells, cardiac
myocytes, skeletal muscle, the kidney and breast cancer
cells and beta cells of pancreatic islets. These sites
of activity of this enzyme allow a tissue specific role
in leading to pathologic states. For instance, in the
beta cells of the pancreatic islets, activation of 12-
LO activity by inflammatory cytokines (e.g., IL-1)
could explain the selective dysfunction and destruction
of the beta or insulin producing cells of the pancreas.
Furthermore, activation or increased expression of the

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12-LO pathway by glucose in the beta cells could
explain the dysfunctional secretion of insulin in the
common form of adult diabetes (non-insulin dependent
diabetes).

5   4.   Factors increasing 12-LO expression and
activity are linked to inflammatory, atherosclerotic,
renal and neoplastic disease.

The factors demonstrated to increase the activity
and expression of 12-LO include, (a) inflammatory

10   cytokines associated with autoimmune disease (Type I
diabetes) atherosclerosis and neoplastic growth such as
interleukin-1β (IL-1), (b) growth factors such as
platelet derived growth factor (PDGF) and angiotensin
II (AII) which have been implicated in accelerated

15   vascular and kidney disease, and (c) hyperglycemia
which has been linked to the microvascular (eye, kidney
and nerve) and microvascular (heart attack, stroke and
peripheral vascular disease) complications of both type
I or type II diabetes.

20   5.   Applicants have found that glucose which
accounts for much of the acquired insulin resistance in
diabetes increases 12-LO activity and expression in all
tissues tested. Therefore, 12-LO activation could
provide a common link between glucose-induced oxidative

25   stress and development of end-organ dysfunction or
damage.

Pursuant to this invention, blockade of the hl 12-
LO expression or enzyme activation provides novel
treatments to prevent these disease states.

30   IDENTIFICATION OF 12-LO
                 IN NORMAL HSMC, HAEC AND MO

Applicants have now evaluated the precise type of
LO present in unstimulated human aortic smooth muscle
cells (HSMC), endothelial cells (HAEC) and monocytes

(MO). Furthermore, since AII can increase the
expression of 12-LO in human adrenal cells, applicants
have also evaluated the effects of AII on 12-LO
regulation in HSMC. Finally, applicants determined

5   whether immunohistochemical analysis of atherosclerotic
lesions demonstrates the presence of a leukocyte type
of 12-LO. The results show that a 12-LO similar to
that found in human adrenal glomerulose is expressed in
the normal HSMC, HAEC and MO. Furthermore, this 12-LO

10   is markedly upregulated by AII in HSMC and is present
in human atherosclerotic lesions.

DESCRIPTION OF FIGURES 1 TO 7

Figures 1A and 1B illustrate RT-PCR analysis of
leukocyte 12-LO RNA in HAEC, HSMC, and MO. Figure 1A

15   illustrates RNA samples that were amplified for 40
cycles with leukocyte specific 12-LO primers Membranes
were hybridized with porcine leukocyte 12-LO
oligonucleotide probe. Lane 1 is a marker, Lanes 2, 5,
8 are negative controls without template, lane 3

20   represents total RNA from HAEC, with porcine leukocyte
12-LO primer, lane 4 with GAPDH primers. Lane 6
represents total RNA from HSMC with porcine leukocyte
12-LO primers, lane 7 with GAPDH primers. Lane 9
represents total RNA from MO with porcine leukocyte 12-

25   LO primers. Lane 10 with GAPDH primers, and lane 11 is
a positive control using the porcine leukocyte 12-LO
cDNA.

Figure 1B illustrates the same RNA samples which
were amplified for 40 cycles with human specific 15-LO

30   primers. Membranes were hybridized with human 15-LO
oligonucleotide. Only the 333 base pair product from
amplification of the 15-LO cDNA (positive control) is
shown.

Figure 2 illustrates the expression of leukocyte
12-LO protein (72 kD) in normal HAEC, HSMC, and MO.
Cytosol fractions from HAEC, HSMC, and MO were
electrophoresed along with authentic porcine 12-LO
5   protein and subjected to Western immunoblotting.

Figure 3A illustrates the effect of AII on 12-HETE
release by HSMC. HSMC were grown to confluency. Serum
was removed and cells were incubated in media 199
containing 0.4% fetal bovine serum (FBS) and 0.2% BSA
10   for 18 hours. Cells were then washed with DME media
and incubated for 20 minutes in DME media containing
0.2% BSA. AII was added to the cells for five and ten
minutes at the concentrations of 10^{-9} and 10^{-8} mol/L.
Media were collected for HETE assay.
15   *$p < 0.05$ vs control $n = 4$

Figure 3B illustrates the effect of AII on cell-
associated 12-HETE levels in HSMC. After collecting
supernatants, cells were washed with ice-cold PBS and
harvested by scraping for the assay of cell-associated
20   HETEs.
*$p < 0.02$ vs control $n = 4$

Figures 4A and 4B illustrate the regulation of
leukocyte 12-LO protein expression by AII in HSMC.
Figure 4A is an immunoblot showing regulation by AII.
25   Figure 4B is a bar graph representation of
densitometric analysis of immunoblot in Figure 4A.
Cells were grown in medium 199 containing 20% FBS and
serum-depleted for 24 hours by placing in medium 199
containing 0.4% FBS and 0.2% BSA. Cells were treated
30   with AII at the concentration of 2×10^{-7} mol/L for 24
to 48 hours. Cells were washed with PBS and harvested
by scraping. Cell pellets were lysed and cytosol
fractions were electrophoresed.

Figures 5A and 5B illustrate the regulation of leukocyte 12-LO mRNA levels by AII in HSMC using RT-PCR. Figure 5A is the autoradiogram of the blot hybridized with 12-LO oligonucleotide probe. Figure 5B

5   is etidium bromide stained agarose gel. Total RNA was extracted from culture HSMC incubated in low serum conditions with AII (2 X 10^{-7} mol/L) for different time-period shown. RNA samples were amplified for 40 cycles with leukocyte 12-LO primers or GAPDH primers.

10   Figures 6A and 6B illustrate microphotographs of a histologic section of an artery obtained from a below-the-knee amputation specimen from a patient with extensive arteriosclerosis. Avidin-biotin complex immunohistochemical technique was used to detect 12-LO

15   with purified specific rabbit anti-sera. Intense staining of endothelial cells (arrowhead), cells present in endothelial thickening (small arrow) and, to a lesser degree, on the smooth muscle cells (larger arrow) were noticed in Figure 6A. Pre-immune rabbit

20   sera was used at the same concentration s negative control (Figure 6B). (X 200 magnification).

Figures 7A and 7B illustrate immunostaining of human coronary lesions using antibody to 12-lipoxygenase. Shown is a cross-section of a human left

25   coronary artery with an advanced atherosclerotic plaque (note the cholesterol crystals in the core region) reacted with either 12-LO antibody (Figure 7A) or pre-immune antisera (Figure 7B). The darkest immunoreactivity is seen in adventitial blood vessels

30   associated with pericytes. The medial smooth muscles cells are also immunoreactive. Lighter immunoreactivity is seen in intimal cells in plaque and non-plaque areas.

EXPERIMENTAL PROCEDURE

The lipoxygenase (LO) pathway has been implicated
in leading to accelerated atherosclerosis. The precise
form of 12-LO expressed in adrenal glomerulose
pancreatic islets is described in application
PCT/US94/00089. This application establishes that a
similar precise type of hl 12-LO is present in
unstimulated human aortic smooth muscle cells (HSMC),
endothelial cells (HAEC) and monocytes (MO). In this
study, the specific reverse-transcriptase polymerase
chain reaction (RT-PCR) method was used to analyze the
type of LO mRNA expressed in normal HSMC HAEC and MO.
In all three cell types, a 333 base pair band was seen
using primers and probes specific for the leukocyte
type of 12-LO suggesting that a leukocyte type of 12-LO
is expressed in these cell types. Western
immunoblotting analysis in cultured HSMC, HAEC and MO
using a polyclonal peptide antibody to leukocyte type
of 12-LO showed a specific 72 kD band which is
identical to the molecular weight of the leukocyte type
of 12-LO. Angiotensin II (AII) added to normal HSMC
increased 12-LO activity and expression.
Immunohistochemical analysis of atherosclerotic lesions
also indicated the presence of a leukocyte type of 12-
LO. These results indicate that a leukocyte type of
12-LO RNA is expressed in HSMC, HAEC and MO. Also, AII
upregulates 12-LO activity and expression in HSMC
supporting a role for this 12-LO pathway in human
vascular disease.

1. Cells and cultures.

HAEC and HSMC were isolated from aortic specimens
obtained from the heart donors in UCLA heart transplant
program. HAEC at passages 5-9 and HSMC at passages 3-7
were used. HAEC were grown in medium 199 containing

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20% FBS supplemented with EC growth supplement (20
mg/ml) and heparin (90 μg/ml). HAEC were identified by
their typical cobblestone morphology, presence of
Factor VIII-related antigen and uptake of acetylated
5   LDL labeled with 1,1'-dioctadecyl-1-3,3,3',3'-
tetramethylindo-carbocyanine perchlorate (Dil-acetyl-
LDL) (19). HSMC were grown in medium 199 containing
20% FBS and identified morphologically and
immunohistochemically using HHF35, which was then
10   visualized by a fluorescently labeled second antibody
or using a biotin-streptavidin complex immunoperoxidase
system (20). Monocytes were obtained from a large pool
of healthy donors by a modification of the Recalde
method (21).
15   HSMC and HAEC monolayers were washed twice with
ice-cold PBS and then processed for RNA extraction or
western analysis as described below. For
hydroxyeicosatetraenoic acid (HETE) assay,
approximately 24 hours prior to an experiment, the
20   medium was replaced with medium 199 containing 0.4% FBS
and 0.2% BSA.
          2.   cDNAs.
          Recombinant Bluescript plasmid containing the cDNA
for human reticulocyte 15-LO was kindly provided by Dr.
25   E. Sigal (Syntex Co., Palo Alto, CA). pUC19 plasmid
containing the cDNA for porcine leukocyte 12-LO was
obtained as described previously (14). Bluescript
plasmid containing the cDNA for human platelet 12-LO
was kindly provided by Prof. Bengt Samuelson
30   (Karolinska Institute, Stockholm, Sweden) (2).
          3.   Oligonucleotide primers and probes for PCR.
          β_2-Macroglobulin oligonucleotides were a kind gift
of Dr. Perrin White (Cornell University Medical
College, New York, NY). Other oligonucleotides

including human glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) oligonucleotides were synthesized
on an Applied Biosystems (Foster City, CA) DNA
synthesizer and were purified by polyacrylamide gel
5   electrophoresis. The sequences of oligonucleotides are
listed in Table 1 and were designed based on known gene
sequences (2,14,22,23) and selected from regions
displaying most divergence between porcine 12-LO and
15-LO sequences (13).

10   **Table 1**

          Primers and probes for amplification and detection

			Sequence (5'-3')	Position
Human 15-LO	5'	primer	AACTCAAGGTGGAACTACCGGAG (SEQ ID NO. 1)	146-168
	3'	Primer	ATATAGTITGGCCCCAGCCATATTC (SEQ ID NO. 2)	453-477
		Probe	AGGCTCAGGACGCCGTTGCC (SEQ ID NO. 3)	306-326
Porcine Leuko- cyte 12-LO	5'	Primer	TTCAGTGTAGACGTGTCGGAG (SEQ ID NO. 4)	145-165
	3'	Primer	ATGTATGCCGGTGCTGGCTATATTT (SEQ ID NO. 5)	451-477
		Probe	TCAGGATGCGGTCGCCCTCCAC (SEQ ID NO. 6)	301-322
human GAPDH	5'	Primer	CCCATCACCATCTTCCAGGAG (SEQ ID NO. 7)	211-231
	3'	Primer	GTTCTCATGGATGACCTTGGC (SEQ ID NO. 8)	475-495

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		Probe	CTAAGCAGTTGGTGGTGCAGG (SEQ ID NO. 9)	446-466
human platelet 12-LO	5'	Primer	GATGATCTACCTCCAAATATG (SEQ ID NO. 10)	472-492
	3'	Primer	CTGGCCCCAGAAGATCTGATC (SEQ ID NO. 11)	610-630
		Probe	GTTTGAGGGCCATCTCCAGAGC (SEQ ID NO. 12)	544-565

        4.   Amplification of reverse transcribed RNA using
10   the polymerase chain reaction (RT-PCR).

        Total RNA from cultured HSMC, HAEC and fresh MO was
extracted with guanidiumthiocyanate-phenol-chloroform
using RNAzol (Cinna/Biotecx Laboratories International
Inc., Texas).   Some RNA samples were treated by RNAse-
15   free DNAse.   3 microgram of total RNA was mixed with the
PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl,
1.5 mmol/L $MgCl_2$, and 0.001% gelatin), 200 μmol/L of each
of the four deoxynucleotide triphosphates, 25 pmol each
of 5'- and 3'-primers, 2 U Avian Myeloblastosis Virus
20   reverse transcriptase (20 U/μl; Life Sciences, St.
Petersburg, FL), and 2.5 U Taq polymerase (Perkin Elmer
Cetus, Norwalk, CT) in a final vol of 50 μl.   In some
reactions, 5 pmol of each 5'- and 3'-primer of β_2
macroglobulin or GAPDH were added as an internal
25   standard.   The samples were placed in a thermal cycler
at 37°C for eight minutes for the RT reaction to proceed.
Then conditions used for PCR were a denaturation step
at 94°C for one minute, annealing at 50°C for two
minutes, and extension at 72°C for two minutes for 20-40
30   cycles.   Blank reactions with no RNA template were
carried out through the RT and PCR steps.   The human 15-
LO cDNA, porcine leukocyte 12-LO cDNA, and human platelet

12-LO cDNA amplifications were carried out by mixing 2-5
ng cDNA plasmid in a 50 μl vol containing 200 μmol/L of
each of the four deoxynucleotide trisphosphates, 25 pmole
5'-and 3'-primers, and 2.5 U Taq polymerase. The
5 conditions for PCR were the same as described before.
        5. Gel analysis and blot hybridization.
        20 μl aliquots of the PCR products were subjected to
electrophoresis in a 1.8% agarose gel in Tris acetate-
EDTA buffer. After staining with ethidium bromide and
10 photographing, the gel was transferred onto a Zeta-probe
membrane (Bio-Rad, Richmond, CA) by capillary blotting.
The oligonucleotides used as probes were labeled at the
5'-end using $[\gamma^{32}P]$ATP and T4 polynucleotide kinase (New
England Biolabs, Beverly, MA) and hybridized with
15 membrane overnight in 6x SSC (1 x SSC contains 0.15 mol/L
NaCl, 0.015 mol/L sodium citrate), 0.5% non-fat dried
milk and 7% SDS at 42°C. Membranes were washed once
in 6 x SSC at room temperature for 15 minutes and then
once at 60°C for 15 minutes. The washing conditions were
20 worked out to distinguish between the PCR products of
human 15-LO from those of porcine leukocyte 12-LO (3).
The filters were exposed to Kodak x-ray film (Eastman
Kodak Co., Rochester, NY) with an intensifying screen
at -70°C. Blots were quantitated using a computerized
25 video densitometer.
        6. Western Immunoblotting.
        Cells pellets were lysed in lysis buffer containing
PBS (pH 7.3), 1% Triton X-100, 1 mmol/L
phenylmethylsulfonyl fluoride, 50 μmol/L leupeptin,
30 and 0.1% sodium dodecyl sulfate (SDS). Lysates were
centrifuged at 10,000 x g for 10 minutes. An aliquot of
the supernatant (cytosol) was saved for protein
estimation and the remainder saved at -70°C for Western
Blot analysis.

SDS polyacrylamide gel electrophoresis (10% running gel, 4% stacking gel) was performed according to the method of Laemmli (24). For Western blotting, gels were equilibrated in transfer buffer (35 mmol/L Tris base, 192

5 mmol/L glycine, and 20% methanol, pH 8.3) and then transferred to nitrocellulose (Hybond, Amersham, Arlington Heights, IL) as described by Towbin et al. (25), in a semidry polyblot apparatus (American Bionetics, Inc., Emeryville, CA) for 40 minutes. The

10 nonspecific sites were blocked with PBS containing 10% of FCS at 4°C overnight. The membranes were then washed twice with PBST (PBS + 0.05% Tween-20) and incubated with primary antibody in PBST containing 1% BSA and 20% (vol/vol) FCS for 2 hours at room temperature. A

15 polyclonal antibody against porcine 12-LO peptide with the sequence of amino acids 646-662 of the porcine leukocyte 12-LO sequence (14) was used. This antiserum was used at 1:100 dilution. In some studies, a polyclonal antibody against human 15-LO kindly provided

20 by Dr. E. Sigal (Syntex Co., Palo Alto, CA) was used. The washed membranes were then incubated for 1 hour with second antibody (goat antirabbit) conjugated with alkaline phosphatase (1:5000; Promega, Madison, WI). Detection was either by color development using substrate

25 mixture (Nitroblue tetrazoleum and 5-bromo-4-chloro-3-indolyl phosphate from Promega) or by chemiluminescence using CSPD substrate and the Western-Light Chemiluminscent detection system (Tropix, Inc., Bedford, MA). Nonspecific binding was evaluated using normal

30 rabbit serum. Western blots were quantitated using a computerized video densitometer (Applied Imagining, Santa Clara, CA; Lynx DNA vision) and values expressed as arbitrary absorbance units.

7.   Measurement of 12-LO products.

These assays were performed using previously
published methods (10,11). Briefly, 12-and 15-HETE are
extracted from supernatants and cells on C18 mini columns
5   (Analytichem International, CA) and measured using our
validated reverse phase gradient HPLC and (RIA) methods.

8.   Measurement of lipoxygenase activity in HSMC.

Confluent HSMC were placed in media plus 10% FCS 24
hours prior to the experiment. The cells were harvested,
10   washed, suspended in 1 ml Tris-HCl buffer (25 mmol/L,
pH 7.7) and then sonicated on ice. The assay mixture
contained in 1.0 ml, 800 μl enzyme (sonicate), 100 μl
$CaCl_2$ (1.5 mmol/L) and 50 μl glutathione (0.5 mmol/L). An
enzyme blank was run simultaneously. The reaction was
15   started at 37°C with 50 μl sodium arachidonate (160
μmol/L Nu Check Prep, Elysain, MN) or 0.25 $\mu Ci^{14}[C]$
linoleic acid (New England Nuclear). After 10 minutes
incubation, the reaction was stopped with 2 ml
isopropanol/1.2% acetic acid followed by 2 ml chloroform.
20   The lower organic layer was filtered and subjected to
HPLC to detect HODEs or HETEs using applicants' gradient-
reverse phase HPLC system (10,11). 12-HETE peak was
identified by UV detection at 237 nM and co-migration
with authentic standard (retention time 18.3 minutes).
25   Peak heights were quantitated using a Shimazu CR5A
integrator. For the identification of radioactive
linoleic acid metabolites, $^{14}[C]$ HODEs, radioactivity in
the fraction co-migrating with the same retention time as
the authentic cold HODEs was quantitated. In this HPLC
30   system, both 9- and 13-HODE have the same retention time
(17.9 minutes).

9.   Immunohistochemistry.

The immunohistochemical method used has been
previously described (26). Briefly, five micron sections

of tissue samples derived from a lower extremity
amputation for peripheral vascular disease or a coronary
artery showing an atherosclerotic plaque were mounted on
Silane (3-Aminoprypyltriethoxysilane, Sigma, St. Louis,
5 MO) coated slides and dried overnight at 60°C. After
deparaffination and dehydration they were placed in a 10
mmol/L citrate buffer solution (pH 6.0) and boiled in a
microwave oven for two periods of 5 minutes each. After
cooling, the sections were twice washed in distilled
10 water. Following a 20 minute incubation in 1% hydrogen
peroxide/methanol, the slides were washed twice in
distilled water and twice in phosphate buffered saline
(PBS). This was followed by blocking with normal horse
serum 1:20 in PBS (Vector Labs). After decanting, the
15 sections were covered with rabbit peptide anti-leukocyte
12-lipoxygenase antisera at 1:1000 dilution and incubated
overnight in a humid chamber at room temperature. After
two washes in PBS the slides were incubated for 40
minutes with biotinylated anti-rabbit IgG (Elite kit,
20 Vector Labs, Burlingame, CA) at 1:600 dilution. After
two additional washes in PBS the sections were incubated
in AB complex (Elite kit, Vector Labs) at 1:200 dilution
for another 40 minutes. The sections were then exposed
to a Diamino benzidine solution for 7 minutes for color
25 development. After two additional washes, the color was
enhanced by incubating the sections in 1% copper sulfate
for 5 minutes. All the steps were performed using an
automatic stainer (Techmate 1000, Biotek Solutions, Santa
Barbara, CA). Sections from the peripheral vessel were
30 washed again and lightly counter stained in 6% Mayer's
hematoxylin, washed, dehydrated and coverslipped. The
sections form the coronary artery were not counter
stained. Control slides were prepared by substituting

anti-12-lipoxygenase with pre-immune rabbit serum at the
same concentration.

　　　10.　Data analysis.

　　　Immunoblots and autoradiograms were analyzed using a
5　computer driven densitometer (Applied Imaging, Santa
Clara, CA; Lynx DNA Vision).　Data shown is
representative of two to three experiments.　Data
generated from AII treatment of HSMC for 12-HETE
synthesis was analyzed using ANOVA for multiple samples
10　using a statistical package on a Macintosh computer
system.　Data is presented as mean ± SE.

　　　　　RESULTS OF EXPERIMENTAL PROCEDURES

　　　Expression of a leukocyte type of 12-LO mRNA in
HAEC, HSMC, and MO.

15　　　The expression of 12-LO mRNA in HAEC, HSMC and MO
was evaluated using a specific RT-PCR method since the
level of detection was below the sensitivity of Northern
analysis.　Figure 1A shows expression of leukocyte 12-LO
mRNA in normal HAEC, HSMC, and MO using a method highly
20　specific for this form of 12-LO mRNA.　The
appropriate 333 base pair band was seen in all three cell
types.

　　　Figure 1B demonstrates RT-PCR analysis of human 15-
LO mRNA expression from the same RNA.　These results
25　reveal no evidence 'for a band characteristic of human 15-
LO.　In a separate experiment, RNA from HAEC, HSMC, and
MO was amplified and probed for the platelet type 12-LO
RNA.　No evidence for a human platelet 12-LO expression
was found (data not shown).

30　　　Expression of hl 12-LO protein in HAEC, HSMC and MO.

　　　To investigate whether a leukocyte type of 12-LO
enzyme was expressed in vascular and circulating MO, the
10,000 xg supernatant proteins were electrophoresed and
subjected to Western analysis using a polyclonal peptide

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antibody derived from a sequence in the porcine leukocyte
type of 12-LO that is homologous to the sequence of 12-LO
found in human adrenal glomerulose. This antibody has
previously been shown to lack cross reactivity to the
5   platelet form of 12-LO and successfully demonstrated the
presence of a leukocyte type 12-LO in human adrenal
cells (3). Figure 2 demonstrates a major 72 kD band from
Western analysis in HSMC, HAEC, and MO. Western analysis
similarly performed using a polyclonal antibody directed
10   against the human 15-LO protein did not demonstrate a
band in the expected molecular weight form these cells
(data not shown). HAEC and MO produced 12-S-HETE as
reflected by HPLC and RIA analysis (HAEC 2386, MO 820
pg/10^6 cells). Results for HSMC are detailed below.
15   Therefore, HSMC, HAEC and MO appear to express a 12-
LO protein which is similar to the leukocyte type of 12-
LO found in porcine tissues and human adrenal
glomerulose.
          Effect of AII on 12-LO activity and expression in
20   HSMC and certain other tissues.
          Another major aspect of this invention is the
discovery that AII increases the activity and expression
of 12-LO mRNA and protein in HSMC. Figure 3A shows
that 5 minute incubation of HSMC with AII at the
25   concentrations of 10^{-8} mol/L and 10^{-9} mol/L in serum free
media stimulates the release of 12-HETE (control: 599 $\pm$
105; AII 10^{-8}M: 1467 $\pm$ 277; AII 10^{-9} mol/L: 1296 $\pm$ 262 pg
per mg of protein). Ten minute incubations with AII also
significantly simulated the release of 12-HETE at the
30   concentration of 10^{-8} mol/L. AII significantly also
increased cell-associated 12-HETE levels in HSMC
(Figure 3B). In other studies, it was found that 12-HETE
levels in response to AII could be reduced by the LO
inhibitor baicalein 10^{-5} mol/L (data not shown).

To examine whether AII induces the 12-LO enzyme
expression in HSMC, cells were treated with AII at the
concentration of 2 x 10^{-7} mol/L for 24 or 48 hours. The
12-LO protein was identified by Western immunoblotting
5   using a specific antibody to purified leukocyte type 12-
LO or a peptide antibody derived from known sequences
present in the human leukocyte type of 12-LO. A distinct
band was detected with a molecular weight of nearly 72 kD
which is the reported molecular weight of the porcine
10   leukocyte-type of 12-LO (Figure 4). A 24 hour incubation
of HSMC with AII in serum free media induced nearly a
seven fold increase in 12-LO protein expression
(Figure 4). In other experiments, AII was added for 48
hours also induced 12-LO expression 4-7 fold (data not
15   shown).

In order to evaluate the specific expression and
regulation of 12-LO mRNA in HSMC, applicants used a RT-
PCR assay that exclusively amplifies the leucocyte type
of 12-LO. The size of the PCR amplified fragment is 333
20   bp for both 12- and 15-LO. Therefore, specific
conditions were used to distinguish leukocyte type 12-LO
and human 15-LO by increasing stringency and raising
washing temperature to 60°C. Figure 5A shows a Southern
blot analysis of RT-PCR amplified products from HSMC
25   serum-deprived for 24 hours and then treated for the
indicated times with AII 10^{-7} mol/L. In this experiment,
very low basal expression of 12-LO is seen. However, in
other experiments in cells from various other donors,
basal 12-LO expression is detectable with PCR at 20-30
30   cycles. AII induces 12-LO mRNA expression starting at
the 12 hour incubation time and the maximum induction is
shown at 36 hour incubation of cells with AII. Figure 5B
shows the ethidium bromide stained agarose gel showing
the amplification of GAPDH as an internal marker. When

PCR conditions were used that were specific for either the platelet type 12-LO or human 15-LO no specific RNA band was detected (data not shown). Therefore, basal serum deprived HSMC show low expression of a leukocyte
5   type 12-LO which is markedly upregulated by AII.

The leukocyte type of 12-LO, unlike the platelet form can also metabolize linoleic acid. Therefore, applicants evaluated whether the HSMC could form 13-HODE, the linoleic acid metabolite of LO action in addition to
10   12-HETE, the product of arachidonic acid metabolism. The accomplish this, applicants performed separate experiments in which the appropriate cytosolic fractions of HSM were treated with either C^{14} linoleic acid or cold arachidonic acid and the LO products of the HSMC were
15   analyzed by a gradient reverse phase HPLC system. Cells labelled with cold arachidonic acid showed HPLC peaks co-migrating with 12-HETE. The peak height in the cell blank sample was 1.5 cm which increased to 3/5 cm in the HSMC sonicate. Cells incubated with C^{14} linoleic acid
20   also produced 13-HODE (695 counts per minute blank to 1038 counts HSMC sonicate).

To evaluate whether a leukocyte type of 12-LO is present in atherosclerotic lesions, immunohistochemistry was performed using the peptide anti-leukocyte type 12-LO
25   antibody. Figure 6A represents a high power section derived from a lower extremity amputation specimen from a patient with peripheral vascular disease. Specific cytoplasmic staining for leukocyte type of 12-LO is evident in both the endothelial and smooth muscle layers
30   of this lesion. The neointimal area also demonstrates staining for leukocyte type 12-LO. Figure 6B represents a high power section from the same lesion stained with pre-immune antisera. This section demonstrates minimal background staining suggesting that the staining for

leukocyte type 12-LO in Figure 6A is specific. Figure 7
is a representative section from a left human coronary
artery showing an advanced atherosclerotic lesion.
Figure 7A shows definite staining for 12-LO protein in
5   the smooth muscle and adventitial areas as well as
endothelial cells. Figure 7B represents
immunohistochemistry with the pre-immune antisera showing
very little background staining with this antibody.
       These results demonstrate that a 12-LO RNA and
10   protein similar to that found in porcine leukocytes and
human adrenal glomerulose (3) is also expressed in human
vascular cells and circulating monocytes. Several
approaches were utilized in this current investigation to
support this conclusion. First, a peptide antibody
15   derived from a sequence common to the porcine and human
form of leukocyte type of 12-LO revealed a characteristic
72 kD band in HSMC, HAEC and MO lysates. This antibody
does not cross react with the platelet form of 12-LO but
has partial cross reactivity with human 15-LO (3).
20   Second, a highly specific RT-PCR procedure was used to
detect 12-LO mRNA in these cell types. In a previous
study, it was demonstrated using this technique that a
leukocyte type of 12-LO was the exclusive type of 12-LO
seen in human adrenal glomerulose and U937 cells (3). In
25   the present study, a specific 333 base pair amplified
mRNA product was found in unstimulated HSMC, HAEC and MO
when appropriate leukocyte type 12-LO primers and probe
were utilized. Thirdly, in all three cell types, the 12-
LO product 12-S-HETE was formed as reflected by HPLC and
30   specific RIA. The cytosol from HSMC reacted with both
arachidonic and linoleic acid to produce either 12-HETE
or 13-HODE respectively. This reaction is characteristic
of a leukocyte type of 12-LO and not the platelet 12-LO

which only reacts with arachidonic acid to produce 12-
HETE.

The human 15-LO originally cloned from the
reticulocyte and found in human trachea is highly
5   homologous (86% sequence homology) to the porcine
leukocyte type of 12-LO (14). The PCR technique utilized
here can distinguish between the leukocyte 12-LO and the
15-LO (3). The specificity of this approach was
demonstrated using the 12-LO and 15-LO cDNA as templates
10   for amplification (3). Therefore, the Southern blot
hybridization using the leukocyte 12-LO probe provides
the strongest evidence that the band seen reflects a 12-
LO and not a 15-LO amplified product. These results are
in agreement with previous studies showing no detectable
15   15-LO mRNA in basal or stimulated human endothelial or
non-stimulated mononuclear cells (27). However, 15-LO
mRNA and protein has been found in macrophage rich areas
of atherosclerotic vascular lesions and in IL-4
stimulated monocytes (28) suggesting that 15-LO can play
20   a role in advanced atherosclerotic and immune mediated
vascular disease.

Increasing evidence also suggests that a 12-LO
enzyme plays an important role in AII-induced actions in
several additional tissues. Studies suggest that the 12-
25   LO pathway of arachidonic acid can mediate AII-induced
aldosterone synthesis in rat and human adrenal
glomerulose cells (11,12). Furthermore, recent data
indicates that AII-induced adrenal cell proliferation is
mediated at least in part by activation of a 12-LO
30   enzyme. Additional studies in the rat have implicated
the 12-LO pathway in the vasoconstrictive and renin-
inhibitory actions of AII (29). The aorta has the
capacity to produce LO products including 12 and 15-HETE
(30). Recent data has revealed that both AII and high

glucose can up-regulate the leukocyte type of 12-LO in
cultured porcine aortic smooth muscle cells (31).

   AII has major effects on vascular smooth muscle cell
growth in vitro and in vivo (1,7, 32-34). In a recent
5 report, it was found that a relatively selective 12-LO
inhibitor but not a cycloxygenase inhibitor could
completely prevent AII-induced hypertrophic responses in
cultured porcine vascular smooth muscle cells (35).
Furthermore, 12-HETE induced similar increases in protein
10 and fibronectin content of these vascular smooth muscle
cells as AII (36). The 12-LO pathway and its product 12-
HETE has also been implicated in vascular smooth muscle
cell migration (9). 12-HETE at concentrations as low as
$10^{-12}M$ have been shown to lead to smooth muscle cell
15 migration. Additional studies have demonstrated that 12-
LO products can activate specific isoforms of protein
kinase C and oncogenzes including ras, c-fos and jun
(36-38).

   Increased 12-LO activity and expression by AII may
20 therefore be a previously unrecognized mechanism for AII-
induced hypertensive and atherosclerotic vascular disease
in humans. Accordingly, another important aspect of this
invention entails blockade of the 12-LO pathway as a
novel therapeutic modality to reduce AII related
25 cardiovascular disease.

   The 12-LO pathway in the human vascular wall and
monocytes may participate in other mechanisms related to
the development or progression of atherosclerotic
vascular disease. Recent evidence has implicated a LO
30 pathway in oxidative modification of LDL in the vascular
wall (39-41). It is now clear that HAEC, HSMC or
monocytes have the capacity to convert native LDL to
minimally modified LDL which has a greater
atherosclerotic potential. Of interest is the data

showing that cholesterol loading of macrophages primarily
leads to increased production of 12-HETE (42). A recent
report has now demonstrated that both the leukocyte type
of 12-LO and 15-LO can similarly oxidize lipoproteins
5 (42). Interestingly, this same report showed a lack of
ability of the platelet 12-LO to oxidize lipoproteins.
        To provide additional evidence for the presence and
localization of a leukocyte 12-LO in human vessels,
immunohistochemical analyses of two atherosclerotic
10 lesions were performed. The results provide
immunohistochemical evidence that a leukocyte type of 12-
LO is particularly expressed in the endothelial and
smooth muscle cells of an atherosclerotic lesion
supporting a potential role of this pathway in the early
15 progression of atherosclerotic vascular disease.
Previous studies have shown that vascular tissues and
monocytes have LO activity (42-44).

              Pathways by which AII and 12-HETE Function

        It has now been discovered that AII and 12-HETE
20 effect changes in cells by stimulating mitogen activated
protein (MAP) kinase activity. Specifically, AII and 12-
HETE function by activating transcriptional activity of
fos via ERK (extracellular regulated kinases) (e.g., ERK
1 ($P44^{MAPK}$), ERK 2 ($P42^{MAPK}$) and ERK3 ($P62^{MAPK}$)), by
25 activating jun via JNK (cJun kinases or stress activated
kinases) and/or by activating JAK (Janus kinases e.g.,
JAK1 and JAK2). More specifically, AII and 12-HETE
activate P-21 activated kinase (PAK), which has been
implicated as a key upstream signal for JNK activation.

30   Experimental Procedures

ERK Activity: ERK activity was evaluated by the
substrate-SDS-polyacrylamide gel method described in
Anal. Biochem., 183:139-143 (1989). Confluent cells in
100 mM dishes were made quiescent in serum-free medium

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and then treated with agonists for various time periods.
The cells were then lysed in lysis buffer (1% NP40, 1%
sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 50 mM Tris,
10 mM EDTA, 1 mM EGTA, aprotinin, 100 mg/ml leupeptin,
5   0.1 mM PMSF and 1 mM sodium orthovanadate, pH 7.4).
Lysates were centrifuged to pellet nuclei and the cell
extracts (15-20 mg protein) were subjected to
electrophoresis on SDS-polyacrylamide gels (10%)
containing myelin basic protein (0.5 mg/ml) as an ERK
10 substrate. The SDS was then washed, followed by
denaturation, renaturation and protein phosphorylation on
the gel with [^{32}P] ATP.

JNK Activity: The plasmid pGEX-cJun 1/79 (Dept.
Pharmacol. UCSD, La Jolla) is a GST-cJun (1-79)
15 expression vector encoding amino acids (1-79) of cJun.
The GST fusion protein expression vector was transformed
into E. Coli. Protein was induced with 0.1 mM IPTG and
purified by affinity binding to glutathione-agarose
beads. Unstimulated or stimulated cells were lysed into
20 WCEB (25 mM HEPEs, pH 7.7, 03.M Nacl, 15 mM MgCl$_2$, 02.mM
EDTA and 0.1% triton X-100). About 50 µg protein extract
was incubated with GST-cJun+GSH agarose overnight at 4°C.
Phosphorylation was carried out at 30°C for 20 minutes in
the presence of 20 mM HEPES, pH 7.5, 20 mM
25 b-glycerophosphate, 10 mM p-nitrophenolphosphate, 10 mM
MgCl$_2$, 10 mM DDT, 50 µg Na$_3$VO$_4$, 20 µM ATP (cold) and about
0.5 µl of new gamma ^{32}P-ATP. After boiling with
electrophoresis sample buffer, the supernatants were
analyzed on 12% SDS-PAGE. JNK activity was also measured
30 by immunoprecipitation method using an anti-JNK antibody
(Parmingen Co., San Diego) and then activities were
measured using 2 µg of GST-cJun (1-79) as substrate as
described in Cell, 81:1147-1157 (1995).

PAK Activity: Cell lysis and PAK activity measurement were performed as described in Science, 269:221-223 (1995).   Cells were lysed on ice in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 10 mM Na pyrophosphate, 1%

5   NP-40, 2.5% glycerol, and 1 mM Na_3VO_4 (pH 7.5), containing protease inhibitors PMSF, leupeptin and aprotinin.   Cell were centrifuged for 10 minutes at 1000 g.   For immunoprecipitation, the supernatants were incubated with anti-PAK1 or anti-PAK2 antibody (1:25) (Dept. Immuno. &

10   Cell Biology, Scripps Res. Instit., La Jolla, CA) for 2 hours at 4°C followed by incubation with 60 μl of 1:1 protein A beads for 60 minutes, then 5 X 1.0 ml lysis buffer washes and 2 washes with kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$ 0.2 mM dithiothreitol, 2 μg

15   myelin basic protein and 14 Mμ [r-^{32}P] ATP) for 20 minutes at 30°C.   The reaction was stopped with SDS sample buffer, and results were visualized by SDS-PAGE and autoradiography.

<div align="center">Results</div>

20   It has been discovered that the administration of AII results in biphasic activation of MAP kinase (all of the MAP kinase experiments were conducted in CHO-AT1a cells -- AII receptor AT1a CDNA transfected Chinese hamster ovary cells).   It has been found that AII (10^{-7}M)

25   induces a biphasic stimulation of ERK activity with a first peak of activity at 5 minutes (2-6 fold) and a later peak at 3-4.5 hours (1.5-3 fold) as well as a stimulation of JNK activity which peaks at 30 minutes and remains sustained for 1 hour.   It has also been found

30   that AII (10^{-7}M) induces stimulation of PAK with one major peak at 30 minutes (5 fold).

     It has also been discovered that 12-HETE (10^{-7}M) induces a biphasic stimulation of ERK activity with a first peak at 5 minutes and a second peak at 3-4.5 hours

as well as a biphasic stimulation of JNK activity with
peaks at 30 minutes (2-3 fold) and 3 hours (2.5-5 fold).
It has been found that 12-HETE stimulates JNK activity at
concentrations as low as $10^{-9}M$, as shown in Figure 29.

5   It has further been discovered that 12-HETE induces
stimulation of JAK activity. CHO-AT$_{1a}$ cells were treated
with 12-HETE ($10^{-7}M$) for 10 hours, immunoprecipitated with
a phosphotyrosine antibody and immunoblotted using
specific JAK1 and JAK2 antibodies. As can be seen in

10   Figure 30, 12-HETE increased JAK activity.

Finally, it has been discovered that 12-LO
inhibitors (e.g., CDC and baicalein) dose-dependently
reduce AII- and 12-HETE-induced mitogenic activities.
Thus, the administration of a 12-LO inhibitor decreases AII-

15   and/or 12-HETE-induced MAP kinase activity, thereby
decreasing the effects which AII and/or 12-HETE have on
cell growth and development.

Consistent with this data, one aspect of the present
invention entails therapy for AII- or 12-HETE-induced

20   disease in humans which includes reducing ERK, JNK and/or
JAK activity via the administration of a 12-LO inhibitor.
While not wishing to be bound by a particular theory, it
is believed that 12-HETE increases MAP kinase activity
through upregulation of 12-HETE receptors. Thus, therapy

25   for AII- or 12-HETE-induced disease also includes
reducing mitogenic activity via the administration of a
12-HETE receptor antagonist such as, for example, DuP654.

Inhibition of the 12-LO Pathway

The utilization of various pharmacologic, antisense

30   or ribozyme methods to reduce leukocyte type 12-LO
activity is described in application PCT/US94/00089.
Panaxynol, a polyacetylene compound isolated from ginseng
has been identified as a relatively selective inhibitor

of leukocyte 12-LO (55) and is useful for the purpose of
this invention.

Role of The 12-LO Pathway in
Breast Cancer Cell Growth

5   Example V of application PCT/US94/00089 indicates
that blockage of the 12-LO pathway provides useful human
breast cancer therapy. A further evaluation of the
regulation of 12-LO activity and expression in breast·
cancer cells and tissues confirms that proliferation of
10   breast cancer tissue is inhibited by 12-LO inhibitors.
Specifically, leukocyte-type 12-LO mRNA expression was
studied by a specific reverse transcriptase PCR method in
matched normal uninvolved and cancer involved breast
tissue RNA samples from six patients. It was observed
15   that in each of the six patients, the cancer involved
section showed a much higher level of 12-LO mRNA (340 bp
PCR product) than the corresponding normal section (3-6
fold higher after normalization to the internal control
for PCR, GADPH mRNA 284 bp). 12-LO mRNA levels were
20   also 7- and 11-fold greater in two breast cancer cell
lines, MCF-7 and COH-BR1 compared to the normal breast
epithelial cell line, MCF-10F. In addition, the
proliferation of MCF-7 cells was significantly inhibited
by three LO inhibitors, baicalein (10 μM), CDC (10^{-5}M) and
25   NDGA (30μM), but not by a cyclooxygenase inhibitor,
ibuprofen (10^{-5}M). Treatment of serum-starved MCF-7 cells
with EGF for four hours lead to a dose dependent increase
in the formation of the 12-LO product, 12-HETE (basal 257
$\pm$ 10 pg/10^6 cells; EGF 50 ng/ml 462 $\pm$ 15 pg; EGF 100 ng/ml
30   593 $\pm$ 46 pg, both p<0.001 vs basal). EGF (50 ng/ml) also
led to a marked increase in the levels of the 12-LO
protein (72 kD) as well as 12-LO mRNA at 24 hours.
Hence, activation of the 12-LO pathway appears to play a

key role in basal and EGF-induced breast cancer cell
growth and development.

Role of the 12-LO Pathway
in the Action of Estrogen in Breast Cancer

5   It has now been discovered that estrogen, which has
been linked to breast cancer cell growth and development,
plays a role in activating the 12-LO pathway in breast
cancer cells.

Treatment of cells from the estrogen receptor
10 positive breast cancer cell line, MCF-7, with 17β-
estradiol for 4 hours in a defined serum-free and phenol
red-free medium led to a dose-dependent increase in the
levels of cell associated 12-LO product 12-
hydroxyeicosatetraenoic acid (12-HETE) (Basal 161 ± 29
15 pg/10^6 cells; 17β-estradiol, 5 nM 784 ± 150 pg; 17β-
estradiol, 10 nM 1056 ± 187 pg; both $p<0.001$ vs basal).
This stimulatory effect of 17β-estradiol on 12-HETE was
not observed in the estrogen receptor negative cell line
MDA-MB-231. Treatment of MCF-7 cells with estrogen for
20 22 hours also caused a dose-dependent increase in the
expression of the leukocyte-type 12-LO protein as
examined by immunoblotting with a 12-LO peptide antibody
(2.1-fold and 3-fold increase over basal at 1 nM and 10
nM 17β-Estradiol respectively). Thus, 17β-estradiol
25 increased 12-LO activity and expression in MCF-7 cells.
Hence, activation of the 12-LO pathway appears to play a
key role in estrogen-induced breast cancer cell growth
and development.

Consistent with this data, one aspect of this
30 invention entails therapy to reduce breast cancer cell
growth and development through inhibition of the 12-LO
pathway. Such 12-LO pathway inhibition would, *inter
alia*, reduce the effect estrogen has on breast cancer
cell growth and development.

                    Role of the 12-LO Pathway
                    in The Formation of VEGF

        Applicants have also discovered that the 12-LO
    pathway plays a role in the formation of vascular
5   endothelial growth factor (VEGF). VEGF is an endothelial
    cell-specific mitogen which increases vascular
    permeability and monocyte migration. VEGF appears to be
    a major angiogenic factor for many types of cancer,
    including breast and lung cancer. Further VEGF has been
10 linked to the development of proliferative diabetic
    retinopathy as well as accelerated vascular disease often
    associated with diabetes.
                 Description of Figures 8, 9 and 10
        Figure 8 illustrates the dose-dependent effect of
15 platelet-derived growth factor (PDGF) on vascular
    endothelial growth factor (VEGF) protein (42 kD)
    expression in MCF-7 breast cancer cells. Nearly
    confluent MCF-7 cells were serum starved for 24 hours by
    placing in DME medium + 0.4% FCS and 0.2% BSA. This
20 medium was then freshly replaced and the cells incubated
    for another 24 hours with PDGF. At the end of the
    incubation, the cell monolayers were washed with ice-cold
    PBS, scraped into PBS and pelleted by centrifugation.
    The cell pellets were then lysed and equal amounts of
25 protein (50 μg) subjected to electrophoresis and
    immunoblotting to detect VEGF using a specific antibody
    from Santa Cruz Biotechnology. Detection was by a
    chemiluminiscent technique. It is clearly seen that PDGF
    causes a dose-dependent increase in the expression of
30 VEGF in the breast cancer cells.
        Figure 9 illustrates the effect of epidermal growth
    factor (EGF) and the 12-lipoxygenase product 12-HETE on
    VEGF protein expression in the MCF-7 breast cancer cell
    line MCF-7. MCF-7 cells were treated with EGF and 12-

HETE for 24 hours and VEGF protein identified as
described in the legend to Figure 8. The figure shows
that VEGF protein expression is not only induced by a
breast cell mitogen such as EGF but also by the 12-LO
5   product, 12-HETE. In fact, 12-HETE appears to be more
potent than EGF in inducing VEGF indicating that 12-HETE
has potent angiogenic properties.
        Figure 10 illustrates the effect of a 12-
lipoxygenase product 12-HPETE on VEGF protein (42 kD)
10 expression in an immortalized human aortic smooth muscle
cell line (AIHSMC). The cells were serum starved for 24
hours and then placed in fresh medium along with 12-
HPETE. Cells were incubated for five hours and VEGF
protein was then identified in cell lysed as described in
15 the legend to Figure 8. Lane 3 shows that the 12-LO
product, 12-HPETE causes an increase in the expression of
the angiogenic agent, VEGF, in PVSMC when compared to the
control in lane 1 as indicated in the figure.
        Figures 8 and 9 report data in two human breast
20 cancer cell lines MCF-7 and MDA MB that show that the 12-
LO product 12-HETE at $10^{-7}M$ and $10^{-8}M$ increases VEGF
protein expression. Figure 10 shows that 12-HETE can
increase VEGF protein in HVSMC.
        In addition to the work reflected in Figures 8-10,
25 it has now been discovered that AII, 12-HETE and
hyperglycemia (HG) increase VEGF production in vascular
smooth muscle cells (VSMC). Porcine and human VSMC were
cultured for at least two passages under normal glucose
(NG, 5.5 mM) or under HG conditions. VEGF protein
30 expression was determined by Western blotting and VEGF
mRNA by Northern blots. HG alone increased the level of
VEGF mRNA (2.8-fold) and protein (3.2-fold). In
addition, VEGF protein (45 K) and mRNA (3.7 Kb)
expression were markedly increased by 4 hours of

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treatment with AII (10^{-7}M) in the cells cultured in HG
(2.2- and 1.4-fold resp.). Furthermore, 12-HETE (10^{-8}M
and 10^{-7}M for 4 hours) increased the expression of both
VEGF protein and mRNA in cells cultured under both NG as
5   well as HG conditions. In addition, HG increased the
secretion of VEGF into the medium as measured by a
specific EIA (56.2 ± 4 ng/ml NG vs 73 ± 5 HG, $p<0.02$).
AII and 12-HETE also increased VEGF secretion by 1.24 and
1.4-fold, respectively, as measured by EIA.

10   Consistent with this data, one aspect of this
invention entails therapy to reduce breast and other
cancer metastic potential as well as afflictions
associated with diabetes (e.g., proliferative diabetic
retinopathy and accelerated vascular disease) by reducing
15   VEGF production, for example, through inhibition of the
hl 12-LO pathway.

                   Role of the 12-LO Pathway in
              The Pathogenesis of Type I Diabetes

          This aspect of this invention involves the role of
20   12-LO pathway activation in the pancreatic beta cell
dysfunction or cytotoxicity in response to cytokines
implicated in the pathogenesis of type I diabetes.

              Description of Figures 11, 12 and 13

          Figure 11 illustrates the effects of 12-LO products
25   on DNA synthesis in the insulin-producing rat beta cell
line, RINm5F. DNA synthesis was studied using ^{3}H-
thymidine incorporation (luCi/ml) added for the last six
hours (18-24 hours of experiment). The LO products were
added to the cells in complete growth media for 24 hours.
30   As demonstrated, 12-hydroxyeicosatetraenoic acid (12-
HETE, 10^{-9}M) and 12-hydroperoxyeicosatetraenoic acid (12-
HPETE) reduced ^{3}H-thymidine incorporation reflecting their
effects to decrease DNA synthesis in this beta cell line.

Figure 12 illustrates a western immunoblot of proteins isolated from the insulin producing rat beta cell line, RINm5F showing the effect of interleukin-1β on 12-LO protein expression. In these studies, 25 μg of
5   protein was isolated from the cells grown for 16 hours in reduced serum-containing medium 0.4% alone or along with IL-1β (0.1 ng/ml). The results demonstrate a 2-fold increase in 12-LO protein expression after IL-1 treatment (lanes 2 and 4). Lane 7 shows the 12-LO standard showing
10 the characteristic 72 kD 12-LO protein band using applicants peptide antibody which recognizes the rat, porcine and human forms of leukocyte-type 12-LO.
    Figure 13 illustrates the effects of IL-1β on 12-HETE production in rat islets.
15 Applicants have discovered that direct addition of the 12-LO products 12-S-HETE or 12-HPETE directly decreases cellular growth as reflected by decreased ^{3}H thymidine incorporation or DNA synthesis in the rat pure beta cell line RIN-M5F. As shown in Figure 11,
20 concentrations of 12-LO products even as low as 10^{-9}M decrease DNA synthesis. Figure 12 demonstrates that human IL-1B at 0.2 ng increases leukocyte 12-LO protein expression approximately two fold in these RIN cells. Figure 13 shows that IL-1B at 2.5 ng/ml markedly
25 increases 12-LO activation in freshly isolated rat pancreatic islets as reflected by an increase in immunoreactive release of 12-S-HETE (92 pg/ml/40 islets basal to 250 pg/ml/40 islets.

Role of The 12-LO Pathway in
The Development of Autoimmune Inflammatory
And Atherosclerotic Disorders in Humans

Increasing evidence suggests that cytokines such as
5   IL_1, IL_4 and IL_8 play a role in the development of
autoimmune, inflammatory and atherosclerotic disorders in
humans.

Description of Figures 14-20

Figure 14A illustrates the dose-dependent effect of
10   the cytokine interleukin-1β on 12-LO mRNA expression in
porcine aortic smooth muscle cells (PVSMC). Confluent
PVSMC growth in normal glucose medium was serum depleted
for 24 hours by placing in medium + 0.2% BSA + 0.4% FCS.
This medium was then freshly replaced along with IL-1β
15   and the cells incubated for a further 24 hours. At the
end of the incubation, total RNA was extracted from the
cells using RNA-STAT. This RNA was the subjected to
reverse-transcriptase polymerase chain reaction (RT-PCR)
to detect and quantitate leukocyte-type 12-LO mRNA (333
20   bp PCR product, upper panel) using our well established
techniques. The expression of GAPDH mRNA 284 bp, lower
panel) was used as an internal control for PCR and for
quantitation. The figure clearly shows that IL-1β
treatment leads to a dose-dependent increase in 12-LO
25   mRNA expression (333 bp PCR product) while there is not
much change in the internal control, GAPDH mRNA
expression.

Figure 14B illustrates the effect of the cytokine
IL-4 on 12-LO mRNA expression in PVSMC. The ells were
30   treated for 24 hours with IL-4 and 12-LO mRNA quantitated
as described in the legend to Figure 14A. It is clearly
seen that IL-4 also increases 12-LO mRNA expression
similar to IL-1β.

Figure 14C illustrates the effect of the cytokine
IL-8 on 12-LO mRNA expression in PVSMC. The cells were
treated for 24 hours with IL-8 and 12-LO mRNA quantitated
as described under legend 14A. The figure shows that
5   IL-8 treatment of PVSMC leads to dose-dependent increase
in 12-LO mRNA expression while there is no change in the
internal control, GAPDH expression.
      Figure 15 illustrates the same RNA analyzed for the
internal marker GAPDH.
10   Figure 16 illustrates the effect of IL-4 on
leukocyte-type 12-LO protein expression in PVSMC.
      Figure 17 illustrates the effect of IL-8 on
leukocyte-type 12-LO protein expression in PVSMC.
      Figure 18 illustrates the effect of IL-4 on 12-LO
15   activity in PSMC.
      Figure 19 illustrates the effect of IL-8 on 12-LO
activity in PSMC.
      Figure 20 reflects the upregulation of hl 12-LO by
IL-1, IL-4 and IL-8.
20   In addition to this work in islets, applicants have
now demonstrated that IL_1, IL_4 and IL_8 can increase the
mRNA expression of leucocyte type 12-LO in porcine and
human aortic smooth muscle cells. Furthermore,
applicants have evidence that 12-LO protein expression is
25   similarly upregulated by these cytokines in porcine
vascular smooth muscle. The cells were cultured in DME
(normal glucose) and treated for 24 hours ± cytokines in
medium containing 0.2% BSA and 0.4% serum. Intracellular
12-LO enzyme activity was measured by HPLC, leukocyte-
30   type 12-LO protein expression by immunoblotting and 12-LO
mRNA by a specific reverse transcriptase polymerase chain
reaction (RT-PCR). All three cytokines (2.5 ng/ml)
caused a marked increase in 12-LO enzyme activity 51, 43
and 36% increase in 12-HETE HPLC peak for IL-1, -4 and -8

respectively). Further, all three cytokines (1-5 ng/ml)
each led to a potent dose-dependent increase (2-5 fold)
in 12-LO mRNA expression (340 bp PCR product). Treatment
with these cytokines (0.5-5 ng/ml) also led to an
5 increase (1.4 -2.5-fold) in 12-LO protein expression
72 kD). In addition, all three cytokines (2.5 ng/ml)
could induce a significant increase in PVSMC DNA
synthesis (1.280.08, 1.67 ± 0.11 and 1.3 ± 0.07 fold increase
in ^{3}H thymidine incorporation with IL-1, -4, and -8
10 respectively, p<0.01).

Human vascular smooth muscle cells (HSMC) were also
cultured in DME (normal glucose) and treated for 24 hours
with IL_1, IL_4 and IL_8 in medium containing 0.2% BSA and
0.4% serum. Intracellular 12-LO enzyme activity in cell
15 sonicates was measured by HPLC, leukocyte-type 12-LO
protein expression by immunoblotting and 12-LO mRNA by a
specific reverse transcriptase polymerase chain reaction
(RT-PCR). Treatment of HSMC in low serum medium for 24
hours with IL-1, IL-4 or IL-8 (5 ng/ml) resulted in 7-10
20 fold increases in 12-LO mRNA expression relative to
untreated cells. RNA from the same experiments was also
analyzed for human 12-LO expression by a specific RT-PCR.
No 15-LO mRNA was seen either in the basal or after
cytokine addition.

25   These results suggest that these inflammatory
cytokines have mitogenic effects in VSMC and that they
are potent positive regulators of the 12-lipoxygenase
pathway. Thus enhanced 12-LO activity and expression in
response to these cytokines may be a key mechanism for
30 cytokine-induced VSMC migration and proliferation
observed in atherosclerosis. Figure 14 shows the effects
of IL_1, IL_4 and IL_8 on 12-LO mRNA expression in PVSMC
cultured in normal (5.5 mM) and elevated (25 mM)
glucoside. Figure 15 represents the same RNA analyzed

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for the internal marker gene GAPDH showing all lanes have
similar amounts of internal standard RNA. Figures 16 and
17 show increases in 12-LO protein expression in PVSMC by
IL_4 and IL_8 respectively and Figures 18 and 19 reveal HPLC
5   tracings showing selective increases in 12-LO protein
activity in PSMC treated with IL_4 and IL_8 respectively.
Figure 20 shows evidence that IL_1, IL_4 and IL_8 can
markedly upregulate leucocyte type 12-LO in human aortic
SMC. In these same experiments, applicants were unable
10 to demonstrate 15-LO expression in untreated or cytokine
treated HSMC demonstrating the selective role of 12-LO as
a potential mediator of cytokine action in vascular
smooth muscle.

<div align="center">Role of 12-LO Pathway in The
Pathogenesis of Type I and II Diabetes</div>
15

Insulin dependent diabetes or type I diabetes is an
autoimmune disease resulting in complete destruction of
the insulin producing cells or beta cells in the
pancreatic islet. Cytokines such as IL-1β are likely to
20 be involved in this autoimmune process.

It has been discovered that IL-1β induces 12-LO
protein and mRNA expression in RIN-M5F cells and 12-LO
mRNA expression in rat islets. RIN-M5F cells treated for
16 hours with IL-1β (25, 50 and 100 ng/L) showed a dose
25 dependent two-fold increase in expression of a porcine
leukocyte form of 12-LO demonstrated by Western blots. A
concomitant increase in 12-LO mRNA expression was seen at
this time point using a highly sensitive competitive PCR
assay. These transcriptional and translational events
30 were paralleled by increased 12-LO pathway activity
measured by radioimmunoassay for 12-HETE. Additionally,
an inhibitor of inducible nitric oxide synthase (iNOS),
N-monomethyl arginine (NMMA), was unable to prevent the
IL-1β induced increase in 12-LO protein expression in RIN

M5F cells, supporting the hypothesis that a pathway
independent of inducible nitric oxide (NO) is present.
Separate experiments using purified Sprague-Dawley rat
islets also showed increased expression of 12-LO mRNA and
5   enzyme activity.

In conclusion, 12-LO is a β-cell specific enzyme
regulated at the transcriptional and translational level
by cytokines like IL-1β.

Description of Figures 21-24

10   Figure 21 reflects the increase in 12-LO mRNA
expression in the pancreatic islets of increasingly
diabetic rats.

Figure 22 indicates that 12-LO mRNA expression in
diabetic ZDF rat skeletal muscle is higher than from non-
15   diabetic ZDF rats. The ZDF rat model has been proposed
as an excellent animal model of spontaneous NIDDM (non-
insulin dependent diabetes mellitus).

Figure 23 presents data pertaining to rat-
fibroblasts which overexpress the human insulin receptor.

20   Figure 24 reflects a major change in the HETE/PGI$_2$
ratio in various diabetic groups.

NIDDM is a complex genetic disorder associated with
a reduced ability of insulin to induce glucose transport
in muscle ("insulin resistance") and a relative
25   impairment of glucose-induced insulin secretion in
pancreatic islets.

Consistent with another aspect of this invention,
increased activity or expression of the 12-LO pathway is
recognized as a common mediator of both of these
30   abnormalities, such that blockade of the 12-LO pathway
may prevent development of NIDDM.

The rationale for this statement includes:

1.   Highly relevant data which evidences the
presence of the leukocyte type 12-LO in pancreatic islets

and skeletal muscle. The data in skeletal muscle is new
and highly relevant. The available data in rat skeletal
muscle and in human islet muscle RNA shows 12-LO
expression using PCR analysis.

5   2.   Evidence that 12-LO mRNA expression
progressively increases in rat pancreatic islets from
lean non-diabetic animals, to obese pre-diabetic and
obese diabetic animals (see Figure 21).

         3.   Data that 12-LO products added exogenously to
10   rat pancreatic islets can reduce glucose-induced insulin
secretion (45).

         4.   In vivo data (see Table 2) that urinary 12-HETE
levels are much higher in male diabetic obese ZDF rats (a
model of NIDDM) compared to lean ZDF non-diabetic rats.

15   Interestingly, obese female ZDF rats which are
phenotypically like pre-diabetic humans show intermediate
levels of 12-HETE in urine.

Table 2

Urinary 12-HETE in ZDF Rats

20   pg/total urine vol.

Diabetic Male Obese Ctrl		2022 ± 372
Non-diabetic Female Obese Ctrl		1007
	Female Obese Mg2+	86
Non-diabetic Lean Male		--
25	Lean Female	--

         5.   Data which indicates that 12-LO mRNA expression
in diabetic ZDF rat skeletal muscle is much higher than
levels in skeletal muscle from non-diabetic ZDF rats
(lane 11 vs lane 21 in Figure 22). Interestingly, 12-LO

30   mRNA levels in skeletal muscle are also higher in obese
female ZDF rats that are prone to get diabetes. In this
figure, the 312 bp band is the 12-LO band while the 281

bp band is the 12-LO competitor. This data represents
true competitive PCR analysis of 12-LO mRNA. Applicants
also have data in the ZDF heart that suggests that 12-LO
expression is also higher in diabetic cardiac tissue.

5   In addition to this work, 12-LO RNA and protein
expression in two NIDDM models, ZDF (Zucker diabetic
fatty) rats and GK (Goto Kyoto) rats have been evaluated.
A specific quantitative polymerase chain reaction (PCR)
assay was used to measure 12-LO mRNA expression and

10   Western blotting using an anti 12-LO peptide antibody was
used to evaluate 12-LO protein expression. The GK rat
model of NIDDM demonstrated an increased blood glucose
concentration compared to age-marched Wistar controls
(8.7 ± 0.7 vs 4.8 ± 0.2 mM, $p<0.01$). However, plasma

15   insulin and body weight were similar between the GK and
Wistar rats. 12-LO mRNA expression was 4-fold greater in
heart from GK rats compared to Wistar ($0.48 \pm 0.1 \times 10^5$
molecules per μg RNA in Wistar vs $1.9 \pm 0.4 \times 10^5$ in GK
$p<0.02$). 12-LO mRNA expression in soleus muscle was over

20   5-fold greater in GK vs Wistar rats (0.6 ± 0.1 Wistar vs
3.1 ± 0.76 GK). The ZDF obese rats demonstrated an
increase in blood glucose concentration and weight
compared to the ZDF lean controls (558 ± 75 vs 170 ± 5
mg/dl and 390 ± 7 vs 343 ± 7 gram respectively). 12-LO

25   mRNA was analyzed in the heart, red and white quadriceps
muscle in the diabetic and lean ZDF rats. 12-LO mRNA
expression was increased by 4-7 fold in the diabetic
obese ZDF rats compared to the lean ZDF controls. 12-LO
protein expression was similarly increased in heart

30   tissue (5-fold) in the diabetic ZDF vs the lean ZDF
controls. These data reflect that muscle 12-LO
expression is markedly increased in both lean and obese
rat models of NIDDM.

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6.   Data which indicates that 12-HETE levels in L_6
muscle cells are increased in the presence of glucose. L_6
muscle cells are skeletal muscle cells from rats which
have been used to investigate the mechanisms of insulin
5   action. When L_6 muscle cells are incubated in xylose
(5.5, mM), they are much more responsive to insulin when
compared to cells in regular glucose (5.5 mM) or high
glucose (25 mM). Table 3 below demonstrates higher
levels of 12-HETE release in the media (pg/ml) or in the
10   cells (pg/total cells) in regular glucose (Rg) or high
glucose (HG) conditions. Thus, elevated 12-Lipoxygenase
products, such as 12-HETE, appear to play an important
role in reduced insulin metabolic actions caused by high
glucose.

15   **Table 3**

         Effect of Glucose on 12-HETE in L_6 Muscle Cells

Cells	Condition		
L_6	Xylose	Rg	Hg
pg/ml (n=2)	53.2	101.8	110.3
20 pg/total cells (n=2)	398	472	620

7.   Baicalein, a selective 12-LO inhibitor, can
prevent glucose-induced insulin resistance. To perform
these studies, applicants cultured rat-1-fibroblasts that
have been engineered to contain the human insulin
25   receptor for ten days in high glucose (25 mM). For the
last 24 hours, the cells were cultured with 10^{-6}M
baicalein. The marked bands represent the phosphorylated
beta sub-unit of the human insulin receptor (Figure 23).
As the band becomes lighter, it represents reduced
30   insulin action. Lane 1 represents the insulin receptor
phosphorylation in normal glucose (5.5 mM) vs the reduced
insulin action in lane 5 (25 mM glucose). Lane 6
represents the insulin receptor phosphorylation in 25 mM

glucose when 12-LO pathway was blocked with baicalein
showing restoration of insulin receptor phosphorylation.

In addition to this work, applicants have discovered
that 12-HETE directly inhibits insulin-induced receptor
5   phosphorylation. As shown in Figure 27, a clear increase
in phosphorylation of the 97 kD beta subunit of the
insulin receptor occurs when insulin is present. This is
demonstrated by a darker band at 97 kD in lane 4
(insulin-treated) vs Lane 1 (control, non-insulin-
10   treated). 12-HETE (10^{-7}M concentration) did not directly
alter basal insulin receptor phosphorylation (lane 2).
In contrast, 12-HETE markedly reduced insulin-induced
receptor phosphorylation of the beta subunit of the human
insulin receptor. This is demonstrated by a reduced band
15   intensity in lane 5 (12-HETE 10^{-7}M) vs. Lane 4 (no 12-HETE
addition).

Since insulin receptor phosphorylation is one of the
early important steps in insulin action, these results
suggest that products of the 12-LO pathway can lead to
20   reduced insulin action. This data suggests that
increased expression or activity of the 12-LO pathway
appears to play a key role in the insulin resistance in
non-insulin dependent forms of diabetes.

8.   Data which indicates that glucosamine, a
25   proposed major mediator of glucose toxicity in terms of
reduced insulin action and vascular disease, increases
12-LO product formation in smooth muscle cells. The role
of glucosamine in leading to insulin resistance has
recently been demonstrated in intact animals (Baron et
30   al., J. Clin. Invest., 96:2792-2801 (1995)) and in
insulin responsive tissues in vitro (e.g., muscle --
Robinson et al., Diabetes, 42:1333-46 (1993); and fat --
Marshall et al., J. Biol. Chem., 266:4706-4712 (1991).
Furthermore, glucosamine is thought to be a major

mediator of glucose-induced vascular disease (Daniels et al., Mol. Endocrinol., 7:1041-1048 (1993)). While not wishing to be bound by a particular theory, it is believed that glucosamine impairs insulin-induced glucose

5   uptake by blocking the normal action of Glut 4, the major glucose transporter linked to insulin ability to transport glucose.

       Porcine vascular smooth muscles cells (PVSMC) were cultured in the presence of glucosamine (7.5 mM) for 24

10 hours. New media was then added with glucosamine (7.5 mM) for 25 minutes. The cells and media above the cells were collected and 12-HETE was measured using RIA. A very large increase in 12-HETE release into the media in cells cultured in glucosamine compared to those in normal

15 glucose (6.48 ± 1.2 pg/ml 12-HETE release in normal glucose vs. 20.6 ± 3.6 pg/ml released in glucosamine n=4) was observed. Furthermore, cell associated 12-HETE was higher in glucosamine treated cells (735.9 ± 67 pg/cell incubate normal glucose vs. 1225 ± 112 pg in

20 glucosamine). These data suggest that 12-LO products such as 12-HETE may be important factors leading to insulin resistance.

       9.   Data which indicates that high fat feeding simultaneously leads to impaired insulin action and

25 induction of 12-LO protein expression in muscle. The mouse model used was a transgenic mouse over expressing the Glut 4 transporter (Pfizer Pharmaceuticals). As can be seen in Figure 28, high fat feeding clearly led to impaired glucose tolerance, which is a clear indication

30 that the animals were insulin resistant. Figure 28a shows a higher glucose level at every point on the oral glucose test curve in the fat fed mice than in the control. The bar graph of Figure 28b demonstrates a

significantly greater area under the glucose tolerance
curve in the high fat fed group than in the control.

    In four of the animals on the high fat diet, 12-LO
protein expression in cardiac muscle was evaluated and
5 compared to the levels in animals on the control diet.
12-LO protein expression was measured using 12-LO peptide
antibody and Western blotting. The summary of the data
is shown in Table 4 below. The striking results show
much higher levels of 12-LO protein (using densitometric
10 analysis of blots) in animals on the high fat diet.
These in vivo results suggest that increased 12-LO
expression or activity plays a key role in leading to
insulin resistance.

Table 4

15   **Densitometry Result Comparisons of**
    12-LO Protein Expression in Heart Muscle Western Blot

Mann-Whitney Test

Mann-Whitney U-statistic = 0.000
U' = 16.000
20 Sum of ranks in Control Male = 10.000
Sum of ranks in Fat Male = 26.000

The two-tailed P value is 0.0286, considered significant.

Parameter:	Control Diet Control Male	High Fat Fed Diet Fat Male
Mean:	2.925	12.897
# of points:	4	4
Std deviation:	1.041	14.636
Std error:	0.5203	7.318
Minimum:	1.890	5.390
Maximum:	4.370	34.850
Median:	2.720	5.675
Lower 95% CI:	1.269	-10.388
Upper 95% CI:	4.581	36.183

10.   Data which indicates that high magnesium (Mg)
feeding markedly reduces 12-LO gene expression and 12-
HETE levels.   Mg deficiency has been associated with
experimental and human insulin resistance.   Moreover,
5   increased dietary Mg has been associated with reduced
development of diabetes in ZDF rats as well as in humans,
and Mg supplementation can improve insulin response and
actions in humans with NIDDM.

It has now been discovered that high Mg feeding
10   markedly reduces 12-LO gene expression and 12-HETE
levels.   High Mg diets (Purina 5008 diet containing 1%
Mg) were fed to one group of ZDF obese (diabetic fatty)
male rats while control diets (Purina 5008 plus 0.2% Mg)
were fed to another group.   As can be seen in Table 5,
15   the high Mg feeding group possessed significantly lower
urinary 12-HETE concentrations as measured by RIA
(methods described in J. Clin. Endocrin. Metab., 67:584-
591 (1988) and J. Clin. Invest., 80:1763-1769 (1987))
than the control group.   High Mg feeding also reduced 12-
20   LO mRNA expression in muscle from diabetic ZDF rats.

Table 5

Urinary 12-HETE Excretion
Rate in ZDF Rat Models

ZDF RAT	12-HETE (pg/min)
ZDF lean (n=4)	[a]0.4 ± 0.07
ZDF obese (n=6)	6.12 ± 1.2
ZDF obese (n=6) with h.Mg diet	[b]3.72 ± 0.5

Values are mean ± SE.   n is the number of rats.
30   Values in ZDF lean group and ZDF obese with high
magnesium diet group are different from ZDF obese
group at [a]$p<0.001$ and [b]$p<0.05$ respectively.

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11. Human data shows increased urinary levels of 12-HETE in people with NIDDM. Levels of 12-HETE are particularly high in diabetics showing evidence of proteinuria. These results suggest that 12-LO activation

5 may be involved in renal disease in diabetes.

Vascular tissue from diabetic animals and man metabolize arachidonic acid differently from normals. PGI_2 is a vasodilator, antithrombolic, and renin secretagogue while 12-hydroxyeicosatetraenoic acid (12-

10 HETE) is proinflammatory and inhibits cyclooxygenase (CO). Applicants earlier reported a prostacyclin (PGI_2) deficiency in diabetics with hyporeinemic hypoaldosteronism (HH) (46). Applicants explored the production of the CO product, PGI_2 and the lipoxygenase

15 (LO) product, 12-HETE in NIDDM patients with normal renal function (NR), those with microalbuminuria (MiA0, macroabluminuria (MaA) and HH patients. PGI_2 (6 keto PGF^1) and 12-HETE were measured in urine by HPLC followed by RIA using published methods. Results are:

	PGI_2 (ng/gm Creat)	12-HETE (ng/gm Creat)	Ratio
Controls (N=17)	64 ± 16	43 ± 9	0.7 ± 0.3
Diabetics (NR) (N=8)	64 ± 9	$122 \pm 34*$	2.0 ± 0.5
Diabetics (MiA) (N=14)	75 ± 10	$226 \pm 60*$	3.8 ± 1.3
Diabetics (MaA (N=9)	$48 \pm 7**$	$352 \pm 152*$	8.1 ± 5.4
Diabetics (HH) (N=5)	39 ± 5	$240 \pm 35*‡$	6.8 ± 2.4

‡ From previously stored samples
* $p < 0.01$ vs controls
** $p < 0.05$ vs diabetics

30 This data suggests that (1) an increase in the 12-LO product 12-HETE is observed in all NIDDM which progresses with renal disease; (2) diabetic renal disease with albuminuria is associated with suppression of PGI_2 production; and (3) HH is a disorder of PGI_2 suppression

35 and 12-HETE excess.

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Applicants further study has measured urinary (renal vascular) production of both PGI_2 and 12-HETE in patients with varying degrees of diabetic renal involvement.

In the group of NIDDM patients with normal renal
5   function based on creatinine clearance and urinary albumin measurements, PGI_2 excretion was not different from normal controls. The group with microalbuminuria were divided into those with hypertension and normotensive. However, no difference in PGI_2 excretion
10 was noted. Nevertheless the microalbuminuria group had significantly lower PGI_2 excretion rates. The patients with macroalbuminuria and reduced creatinine clearance similarly had reduced PGI_2 excretion. PGI_2 excretion rates were reduced in the macroabluminuria and HH group.

15   12-HETE values were markedly increased in NIDDM patients with or without microalbuminuria compared with normal controls. 12-HETE excretion values were also significantly increased in the macroabluminuria group as well as in the HH group.

20   Low dose calcium infusions have been previously shown to increase PGI_2, probably via activation of tissue phospholipases. When a three hour infusion of calcium gluconate was administered to normal subjects, there was a highly significant increase in both PGI_2 and 12-HETE.

25 However, when administered to NIDDM patients with microalbuminuria, there was no increase in PGI_2 but a further stimulation of the already increased 12-HETE values. This supports the concept that a defect in prostacyclin formation exists in NIDDM.

30   12-HETE/PGI_2 ratios were calculated as an additional approach to define whether in NIDDM there is an alteration in the LO/CO pathways. As shown in Figure 24, there is a major change in the HETE/PGI_2 ratio in all diabetic groups. The mean value is significantly

increased in NIDDM patients with normal renal function
and is further altered in patients with macroalbuminuria
and HH patients. However, these differences were not
significantly different between the NIDDM groups due to
5 the variability within each group.

        Applicants data appears to exclude renal function
per se, GFR or hypertension as a cause of the deranged
eicosanoid excretion values. While the origin of PGI_2 and
12-HETE in urine has not been fully settled, studies
10 using extrarenal CO inhibitors and lack of excretion of
tracer PGI_2 and 12/15-HETE following systemic injection,
suggests hat the kidney is the major source of these
compounds in urine (47-49). HETEs can be generated in
vascular tissue as well as from inflammatory cells.
15 (50-51). However, there is no evidence for
macrophage/leukocytes infiltration into the kidney in
NIDDM with only incipient glomerular and vascular
disease.

        In agreement with in vitro and animal model studies
20 cited earlier, applicants results suggest that early in
diabetes mellitus, there is fixed prostacyclin production
which falls to lower values with diabetic renal
vascular/glomerular disease. This occurs in a state
where the LO product 12-HETE is increased early in
25 diabetes mellitus prior to development of
microalbuminuria. These observations could be of
considerable importance in the etiology of diabetic
vascular disease since the HETEs are mitogenic
proinflammatory, vasoconstrictive, and stimulate
30 angiogenesis (52-53). With respect to the HH syndrome,
applicants new data suggests that increased HETE
production may be an early abnormality in suppressing PGI_2
formation and renin biosynthesis and secretion. The
etiologic event in diabetes explaining vascular disease

is not known. However, recent studies suggest that
hyperglycemia per se has a number of metabolic
consequences including enhanced eicosanoid generation via
protein kinase C and activation of calcium dependent
5   phospholipases, major mediators of AA release (54).

In summary, whether cause or effect, very early
involvement of the kidney in diabetes is associated with
fixed or suppressed production of prostacyclin, with
increase in the vasculotoxic lipoxygenase product 12-
10   HETE. This conclusion is now suggested by both in vitro
studies and in vivo studies in man. This suggests
pharmacologic intervention early in the diabetic state to
block this derangement.

                    Role of 12-Lipoxygenase Products in
15   Glucose-Induced Monocyte Binding to
                    __Human Aortic Endothelial Cells__

The rate of atherosclerosis is accelerated in humans
with diabetes mellitus (DM). Applicants recently
published evidence that high glucose (HG) exposure of
20   human aortic endothelial cells (HAEC) selectively
increases monocyte (MO) but not neutrophil binding (56).
HG exposure to EC can increase arachidonic acid (AA)
release and lipoxygenase (LO) production formation. In
the current study, applicants evaluated the role of 12-
25   and 15-LO products in MO binding to HAEC. Culture of
HAEC in HG (25 mM) for two passages increased MO binding
compared to cells maintained in 5.5 mM glucose (239 ± 30
cells/field HG vs 111 ± 7, $p<0.01$). Phenidone ($10^{-6}M$), an
inhibitor of the 12-LO pathway (50 percent inhibition of
30   HG-induced binding, $p<0.05$). HG culture of HAEC
significantly increased both 12- and 15-
hydroxyeicosatrienoic acids (HETEs) using applicants HPLC
and RIA methods. 12(S)-HETE added to HAEC cultured
in 5.5 mM glucose increased MO binding (66 ± 6 cells/field

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control vs 114±4 12-HETE 10^{-10}M, p<0.01). Another novel
12-LO product 12(R)-Hydroxyeicosatrienoic acid was even
more potent showing effects on MO binding at 10^{-11} and
10^{-12}M. In contrast, 15(S)-HETE added at concentrations
5 ranging from 10^{-6} to 10^{-12}M did not stimulate MO binding to
HAEC. In summary, (1) elevated glucose increases MO
binding to HAEC and this effect can be reduced by
blockade of the LO pathway; (2) 12 but not 15-LO
products, can increase MO binding. Since a leucocyte
10 type 12-LO is expressed in HAEC, these results support
the role of 12-LO activation in glucose-induced MO
binding to human endothelium.

Applicants have also demonstrated that high fat
"cafeteria" diets increase leukocyte 12-LO in rat hearts
15 and that diabetic (GK) rats have a higher 12-LO in heart
compared to normal (Wistar) animals (see Figures 25
and 26).

DIAGNOSTIC ASSAYS

Application PCT/US94/00089 reports that antibodies
20 would circulate in patients at risk for developing
disease states for which hl 12-LO or its pathway products
such as HETE or 12-HPETE are the etiological agent.
Accordingly, another aspect of this invention includes
assays, e.g., of the ELISA type in which hl 12-LO
25 protein, or a related material such as HETE is utilized
as an immunogen. Such tests are useful to diagnose any
of the disease states mediated by the activation or
expression of hl 12-LO.

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<u>SEQUENCE LISTING</u>

(1)   GENERAL INFORMATION:

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                              Rama Natarajan

5   (ii)   TITLE OF INVENTION: Human Leukocyte 12-
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                   Pathogenesis of Disease States

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          (v)   COMPUTER READABLE FORM:

              (A) MEDIUM TYPE: 3M Double Density 5 1/4"
                   diskette

              (B) COMPUTER: Wang PC

20   (C) OPERATING SYSTEM: MS DOS Version 3.20

              (D) SOFTWARE: Wordperfect

          (vi)   CURRENT APPLICATION DATA:

              (A) APPLICATION NUMBER:

              (B) FILING DATE: 04 May 1995

25   (C) CLASSIFICATION: Unknown

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  (2)   INFORMATION FOR SEQ ID NO: 1:

      (i)   SEQUENCE CHARACTERISTICS:

           (A)   LENGTH: 23

15   (B)   TYPE: Nucleotide

           (C)   STRANDEDNESS: Single

           (D)   TOPOLOGY: Linear

      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AACTCAAGGT GGAACTACCG GAG   23

20 (2)   INFORMATION FOR SEQ ID NO: 2:

      (i)   SEQUENCE CHARACTERISTICS:

           (A)   LENGTH: 24

           (B)   TYPE: Nucleotide

           (C)   STRANDEDNESS: Single

25   (D)   TOPOLOGY: Linear

          (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATATAGTITG GCCCCAGCCA TATT   24

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          (i)   SEQUENCE CHARACTERISTICS:

5   (A)   LENGTH: 20

            (B)   TYPE: Nucleotide

            (C)   STRANDEDNESS: Single

            (D)   TOPOLOGY: Linear

          (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 3:

10 AGGCTCAGGA CGCCGTTGCC   20

   (2)   INFORMATION FOR SEQ ID NO: 4:

          (i)   SEQUENCE CHARACTERISTICS:

            (A)   LENGTH: 21

            (B)   TYPE: Nucleotide

15   (C)   STRANDEDNESS: Single

            (D)   TOPOLOGY: Linear

          (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTCAGTGTAG ACGTGTCGGA G   21

   (2)   INFORMATION FOR SEQ ID NO: 5:

20   (i)   SEQUENCE CHARACTERISTICS:

            (A)   LENGTH: 25

            (B)   TYPE: Nucleotide

            (C)   STRANDEDNESS: Single

            (D)   TOPOLOGY: Linear

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATGTATGCCG GTGCTGGCTA TATTT   25

    (2)   INFORMATION FOR SEQ ID NO: 6:

        (i)   SEQUENCE CHARACTERISTICS:

5   (A)   LENGTH: 22

           (B)   TYPE: Nucleotide

           (C)   STRANDEDNESS: Single

           (D)   TOPOLOGY: Linear

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 6:

10   TCAGGATGCG GTCGCCCTCC AC   22

    (2)   INFORMATION FOR SEQ ID NO: 7:

        (i)   SEQUENCE CHARACTERISTICS:

           (A)   LENGTH: 21

           (B)   TYPE: Nucleotide

15   (C)   STRANDEDNESS: Single

           (D)   TOPOLOGY: Linear

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CCCATCACCA TCTTCCAGGA G   21

    (2)   INFORMATION FOR SEQ ID NO: 8:

20   (i)   SEQUENCE CHARACTERISTICS:

           (A)   LENGTH: 21

           (B)   TYPE: Nucleotide

           (C)   STRANDEDNESS: Single

           (D)   TOPOLOGY: Linear

-58-

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTTCTCATGG ATGACCTTGG C   21

(2)   INFORMATION FOR SEQ ID NO: 9:

(i)   SEQUENCE CHARACTERISTICS:

5   (A)   LENGTH: 21

(B)   TYPE: Nucleotide

(C)   STRANDEDNESS: Single

(D)   TOPOLOGY: Linear

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 9:

10   CTAAGCAGTT GGTGGTGCAG G   .   21

(2)   INFORMATION FOR SEQ ID NO: 10:

(i)   SEQUENCE CHARACTERISTICS:

(A)   LENGTH: 21

(B)   TYPE: Nucleotide

15   (C)   STRANDEDNESS: Single

(D)   TOPOLOGY: Linear

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GATGATCTAC CTCCAAATAT G   21

(2)   INFORMATION FOR SEQ ID NO: 11:

20   (i)   SEQUENCE CHARACTERISTICS:

(A)   LENGTH: 21

(B)   TYPE: Nucleotide

(C)   STRANDEDNESS: Single

(D)   TOPOLOGY: Linear

-59-

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 11:

    CTGGCCCCAG AAGATCTGAT C   21

        (2)   INFORMATION FOR SEQ ID NO: 12:
            (i)   SEQUENCE CHARACTERISTICS:
5   (A)   LENGTH: 22
             (B)   TYPE: Nucleotide
             (C)   STRANDEDNESS: Single
             (D)   TOPOLOGY: Linear
        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 12:
10   GTTTGAGGGC CATCTCCAGA GC   22

CLAIMS:

1.   Isolated and purified human 12-lipoxygenase from human tissue, said isolated and purified human 12-lipoxygenase being distinct from that found in human platelets and distinct from human 15-lipoxygenase.

2.   The isolated and purified human 12-lipoxygenase of claim 1, wherein said 12-lipoxygenase is from human vascular smooth muscle cells, adrenal cells, endothelial cells or monocytes.

3.   A method for diagnosing a disease state in a patient in which the 12-lipoxygenase of claims 1-2 or 12-HETE is an etiological agent which comprises:

(i)   obtaining a physiological specimen from
5   said patient; and

(ii)   determining whether the etiological agent or antibodies to said etiological agent are present in said specimen.

4.   The method of claim 3 in which said disease state is an inflammatory or autoimmune condition atherosclerosis, cancer growth, cancer metastasis, dysfunctional secretion of insulin in non-insulin
5   dependent diabetics, glucose-induced oxidative stress or the development of end-organ dysfunction or damage.

5.   The method of claim 4, wherein said disease state is atherosclerotic vascular disease, human breast cancer, or Type II diabetes.

6.   A method for treating a patient suffering form a disease state in which hl 12-lipoxygenase or 12-HETE is an etiological agent which comprises administering to said human patient an hl 12-lipoxygenase inhibitor in an
5   amount effective to retard or inhibit expression of said hl 12-lipoxygenase.

7.   The method of claim 6, wherein said disease state is Type II diabetes or breast cancer.

-61-

8.   The method of claim 6, wherein said disease
state is non-insulin dependent diabetes mellitus and the
human 12-lipoxygenase inhibitor is administered in an
amount therapeutically effective to enhance the ability
5   of insulin to induce glucose transport in muscle and the
secretion of insulin in pancreatic islets.

9.   The method of claim 6, in which said inhibitor
is NDGA, CDC, panaxynol, biacalein, pioglitazone,
aminoguanidine or a ribozyme which cleaves hl 12-
lipoxygenase mRNA.

10.   A method for inhibiting or reducing vascular
endothelial growth factor production in a human patient
which comprises administering to said patient an hl 12-
lipoxygenase inhibitor in an amount effective to retard
5   or inhibit the expression of hl 12-lipoxygenase.

11.   A method for the treatment of a human patient
suffering from a cytokine mediated autoimmune,
inflammatory or atherosclerotic disorder which comprises
administering to said patient a human 12-lipoxygenase
5   inhibitor in an amount therapeutically effective to
mediate the action of said cytokine on human vascular
smooth muscle.

12.   The method of claim 11 in which said cytokine
is interleukin 1, interleukin 4 or interleukin 8.

13.   A method for reducing monocyte binding to human
endothelial cells which comprises administering to a
patient in need thereof an inhibitor of human 12-
lipoxygenase in a therapeutically effective amount.

14.   The method of claims 10, 11, 12 or 13, wherein
said inhibitor is NDGA, CDC, panaxynol, baicalein,
pioglitazone, aminoguanidine or a ribozyme which cleaves
hl 12-lipoxygenase mRNA.

15.   A method for generating lipid mediators which
activate signal transduction pathways associated with

inflammatory and autoimmune conditions, atherosclerosis,
cancer growth and metastasis, said lipid mediators being
5   hydroperoxides, kinases, mitogen activated kinases,
transcription factors, and oncogenes which method
comprises administering to a mammal in need thereof a
therapeutically effective amount of arachidonic or
linoleic acid, wherein said human 12-lipoxygenase
10   utilizes said acid to generate such a lipid mediator.

       16.   The method of claim 15 in which said lipid
mediator is a hydroperoxide, a mitogen activated kinase
or NFkB.

       17.   A method for increasing the activity and
expression of human 12-lipoxygenase which comprises
administering to a human patient a therapeutically
effective amount of an inflammatory cytokine, a growth
5   factor or angiotensin II.

       18.   The method of claim 17 in which said
inflammatory cytokine is interleukin-1.

       19.   The method of claim 17 in which said growth
factor is platelet derived growth factor.

       20.   A method for increasing the activity and
expression of 12-lipoxygenase mRNA and protein in human
aortic smooth muscle cells which comprises treatment of
said cells with angiotensin II in an amount effective to
5   increase said activity and expression.

       21.   A method for the reduction of angiotensin II
related cardiovascular disease which comprises
administration to a patient in need thereof a
therapeutically effective amount of a drug effective to
5   blockade the human 12-LO pathway.

       22.   A method for inhibiting the proliferation of
breast cancer tissue in a human patient which comprises
administering to said patient a therapeutically effective

amount of a drug which inhibits human 12-LO expression or
activation.

5

23.   The method of claim 22 in which said human 12-
lipoxygenase inhibitor is NDGA, CDC, panaxynol,
baicalein, pioglitazone, aminoguanidine or a ribozyme
which cleaves hl 12-lipoxygenase mRNA.

24.   The method of claim 22 in which the breast
cancer cell growth and development is basal, epidermal
growth factor-induced or estrogen induced.

25.   A method for mediating breast cancer cell
growth and development which comprises administering to a
patient in need thereof a therapeutically effective
amount of a human 12-lipoxygenase pathway inhibitor.

26.   The method of claim 25 in which said human 12-
lipoxygenase inhibitor is NDGA, CDC, panaxynol,
baicalein, pioglitazone, aminoguanidine or a ribozyme
which cleaves hl 12-lipoxygenase mRNA.

27.   The method of claim 25 in which the breast
cancer cell growth and development is basal, epidermal
growth factor-induced or estrogen induced.

28.   A method for monitoring cytokine induced
vascular smooth muscle cell migration and proliferation
observed in an atherosclerosis which comprises measuring
the change in human 12-lipoxygenase mRNA or protein

5   expression by said cells upon treatment with a cytokine
wherein an increase in said expression is indicative of
said cell migration and proliferation.

29.   A method for treating a patient having a
disease state in which 12-HETE is an etiological agent
which comprises decreasing mitogenic activity in said
patient.

30.   The method of claim 29, wherein the decrease in
mitogenic activity results from reducing ERK, JAK and/or
JNK activity in said patient.

31. The method of claim 30, wherein JNK activity is reduced by decreasing PAK activity.

32. The method of claim 29, wherein the mitogenic activity is decreased by administering a therapeutically effective amount of a 12-LO inhibitor to the patient sufficient to decrease mitogenic activity.

33. The method of claim 29, wherein the disease state is Type II diabetes or breast cancer.

34. A method for treating a patient having a disease state in which VEGF is an etiological agent which comprises decreasing the amount of VEGF in said patient.

35. The method of claim 34, wherein the disease state is proliferative diabetic retinopathy or accelerated vascular disease associated with diabetes.

36. The method of claim 34, wherein the amount of VEGF is decreased by administering to the patient a therapeutically effective amount of a 12-LO inhibitor sufficient to decrease VEGF in the patient.

37. A method for increasing insulin receptor phosphorylation in patients having Type II diabetes which comprises administering to the patient a therapeutically effective amount of a 12-LO inhibitor sufficient to
5   inhibit 12-LO pathway products from inhibiting insulin receptor phosphorylation.

38. A method for reducing 12-LO gene expression and 12-HETE levels in a patient which comprises administering to the patient a therapeutically effective amount of Mg.

FIG. 1A
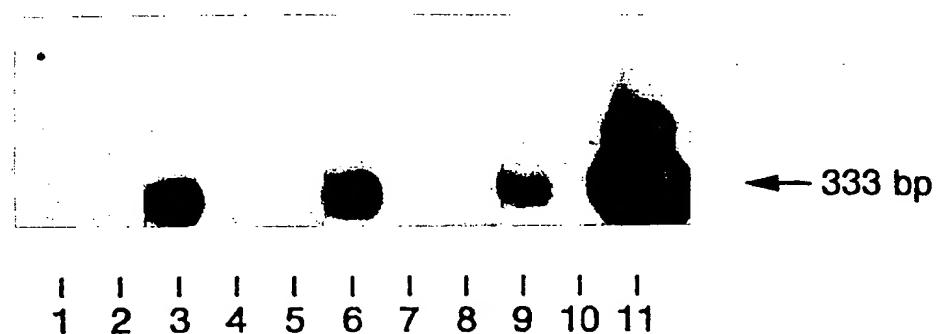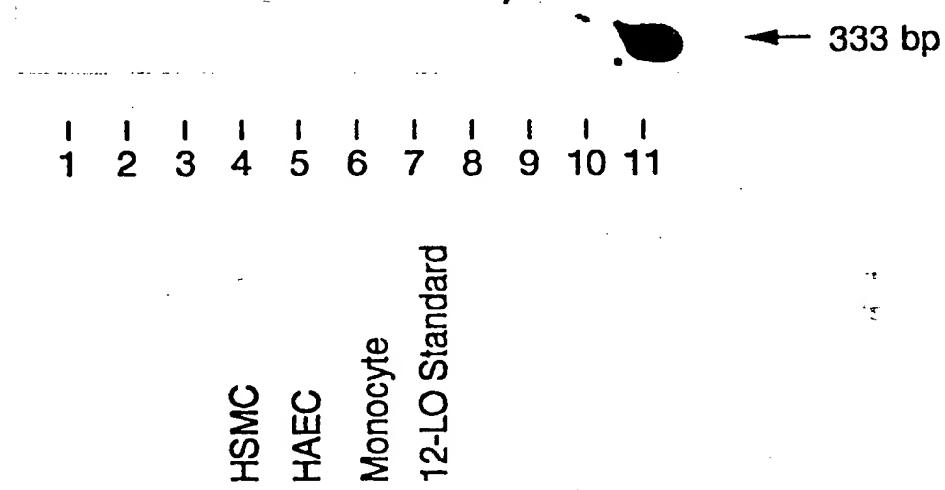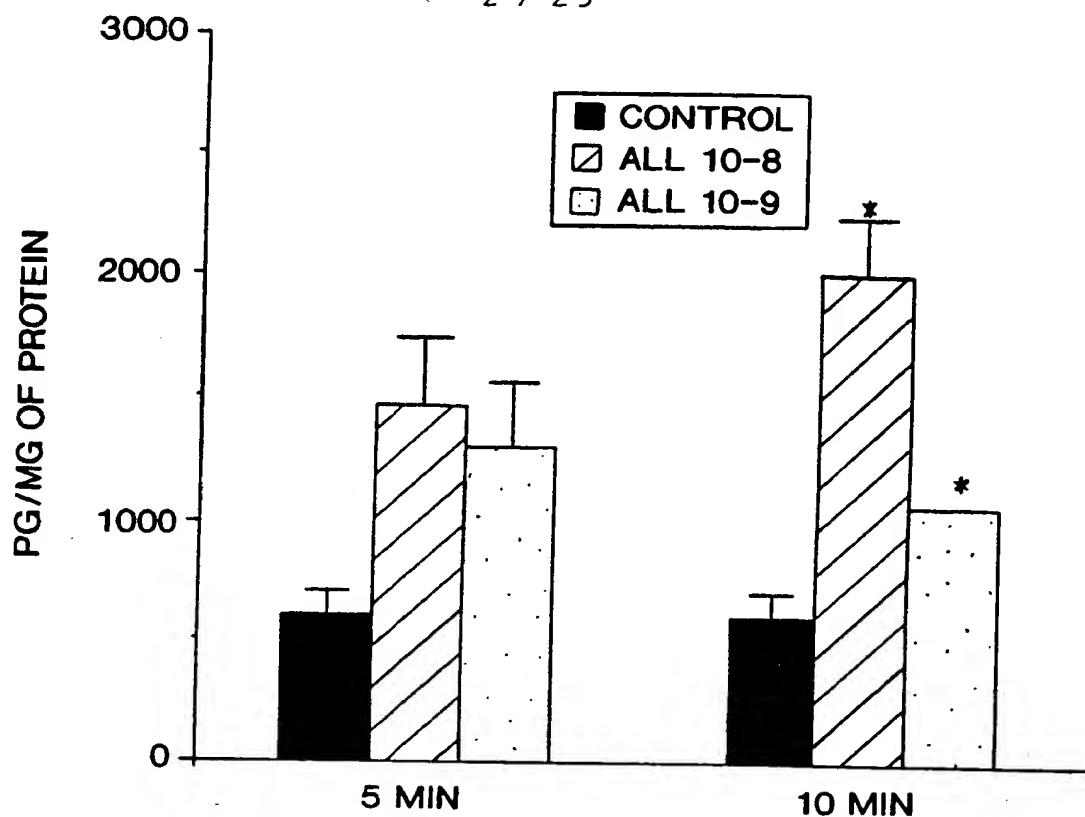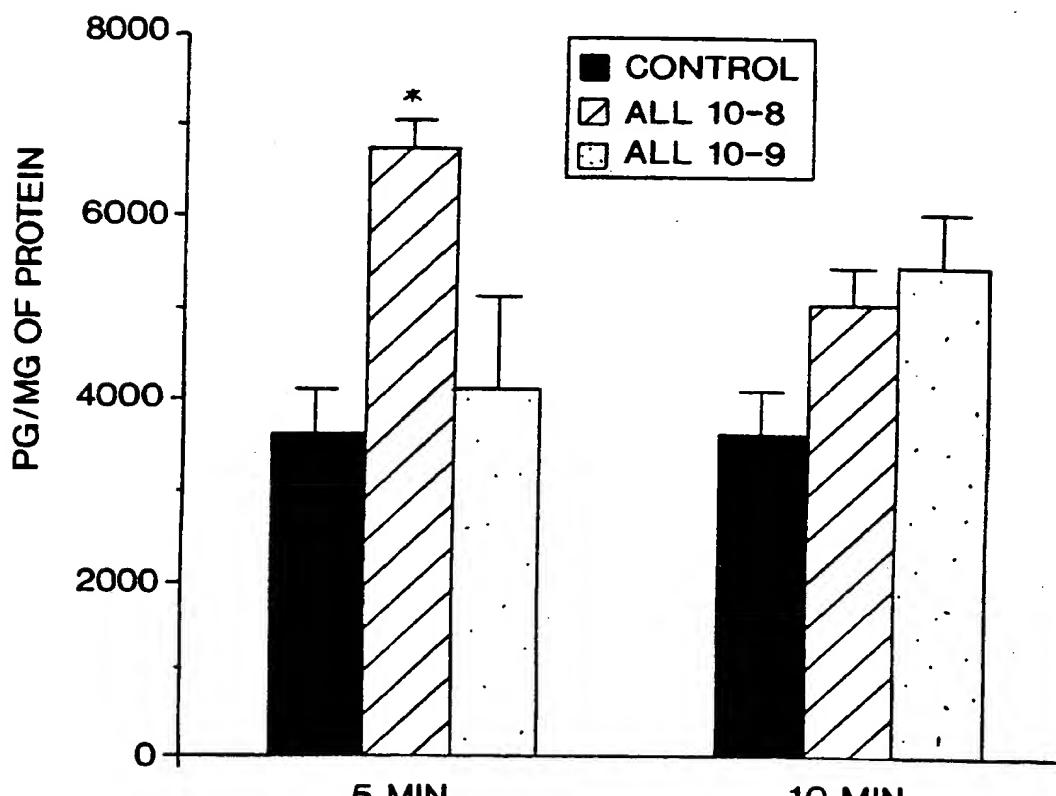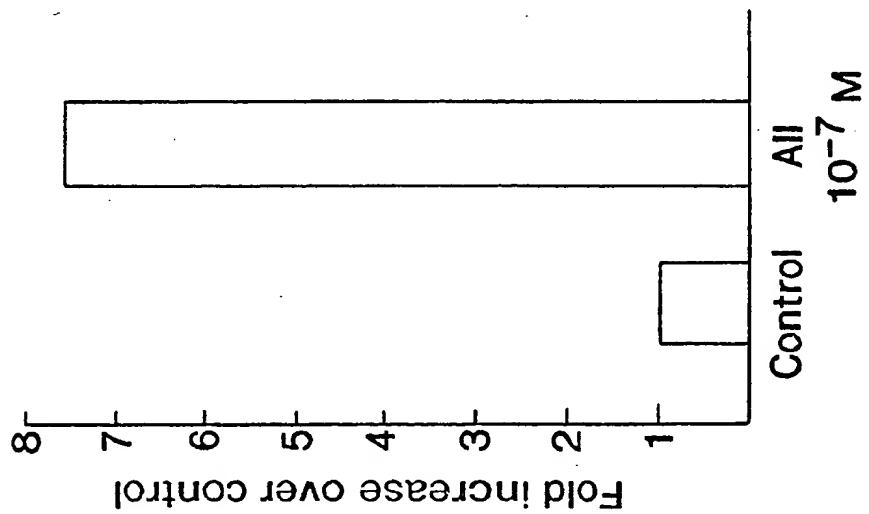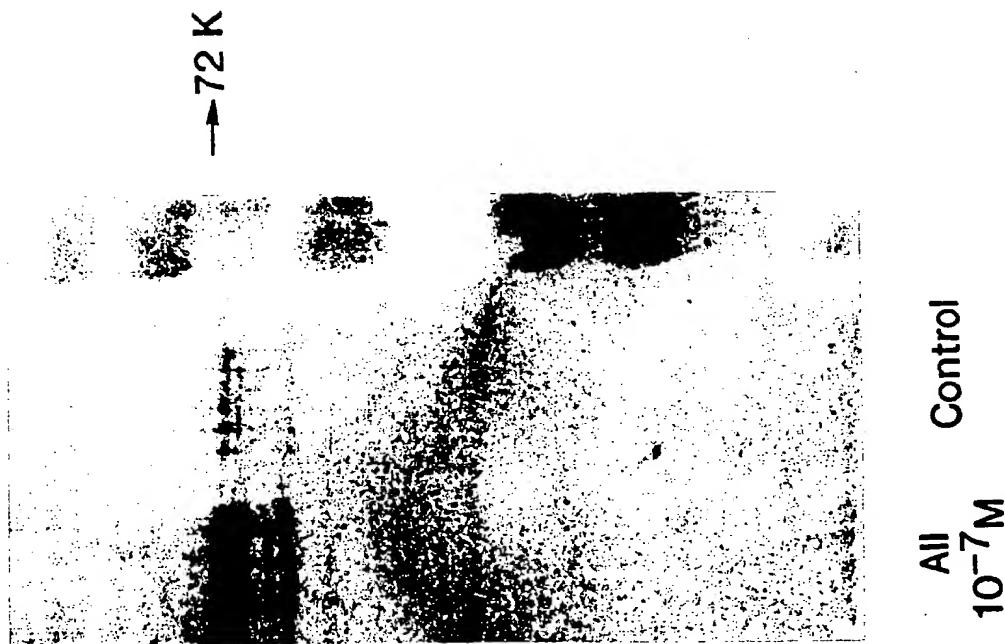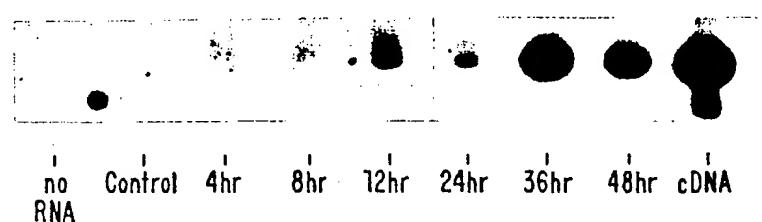
← 333 bp

1 2 3 4 5 6 7 8 9 10 11

FIG. 1B

← 333 bp

1 2 3 4 5 6 7 8 9 10 11

HSMC
HAEC
Monocyte
12-LO Standard

FIG. 2

← 72 kD

2 / 23

FIG. 3A

FIG. 3B

FIG. 4B

FIG. 4A

no Control 4hr 8hr 12hr 24hr 36hr 48hr cDNA
RNA

FIG. 5A

Marker no   Control   4hr   8hr   12hr   24hr   36hr   48hr   cDNA
        RNA
                    * GAPDH primer

FIG. 5B

FIG. 6A

FIG. 6B

FIG. 7A

FIG. 7B

Control

PDGF 10^{-10}M

PDGF 10^{-9}M

—42 kD

FIG. 8

Control

EGF (50 ng/ml)

12-HETE (10^{-7}M)

—42 kD

FIG. 9

SUBSTITUTE SHEET (RULE 26)

FIG. 10

FIG. 11

FIG. 12

FIG. 13

FIG. 14A

FIG. 14B

FIG. 14C

FIG. 15

FIG. 17

FIG. 16

FIG. 18

FIG. 19

333 bp

NO RNA
CONTROL
IL-1
IL-4
IL-8
cDNA
CONTROL/GAPDH
IL-1/GAPDH
IL-4/GAPDH
IL-8/GAPDH

FIG. 20

FIG. 21

FIG. 22

1   2   3   4   5   6   7   8

5.5mM   2.5mM   2.5mM glucose
glucose   glucose plus baicalein
                            $10^{-6}M$

FIG. 23

←--75kD
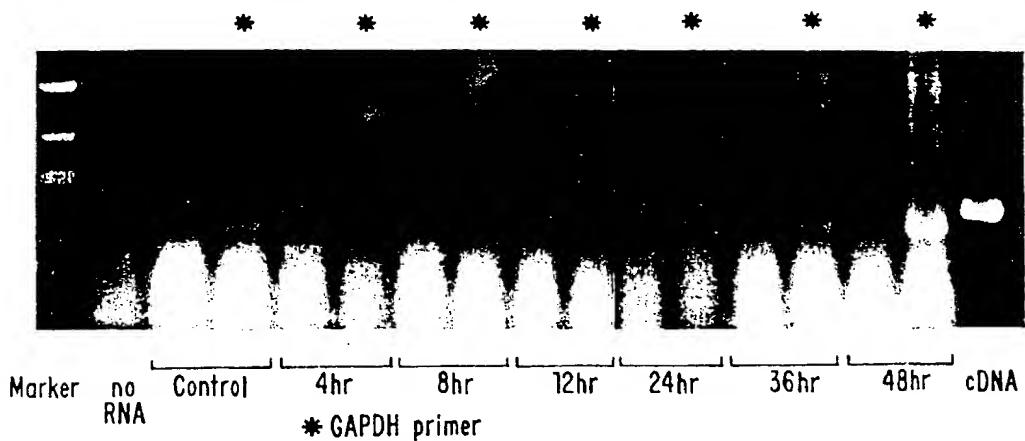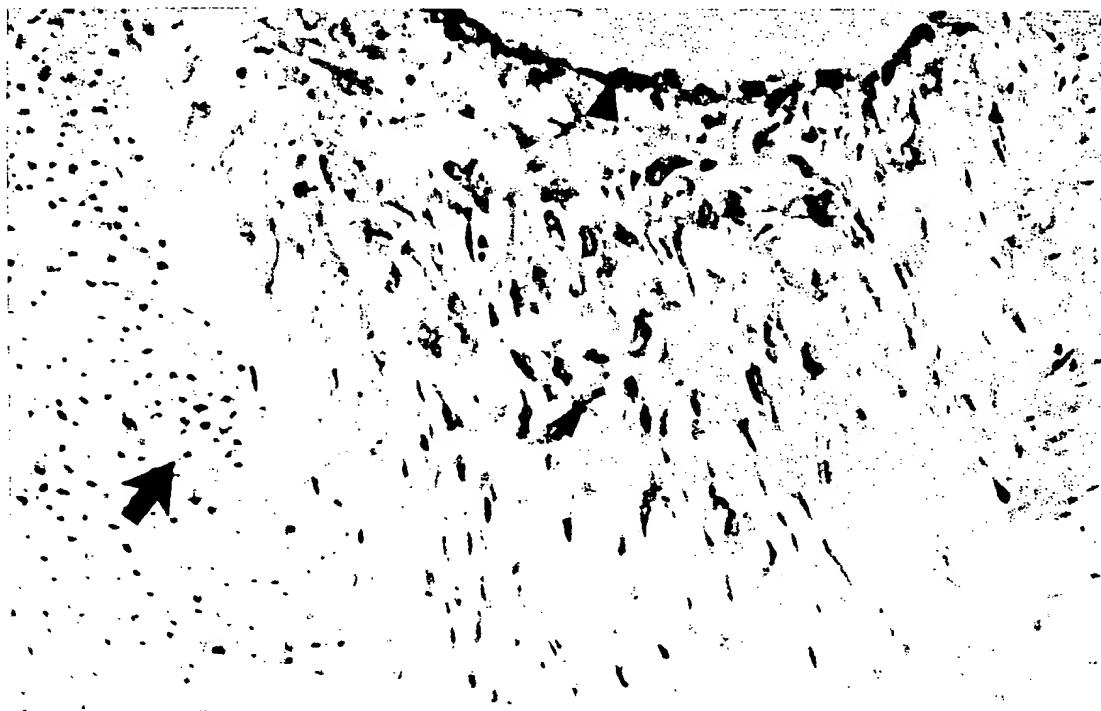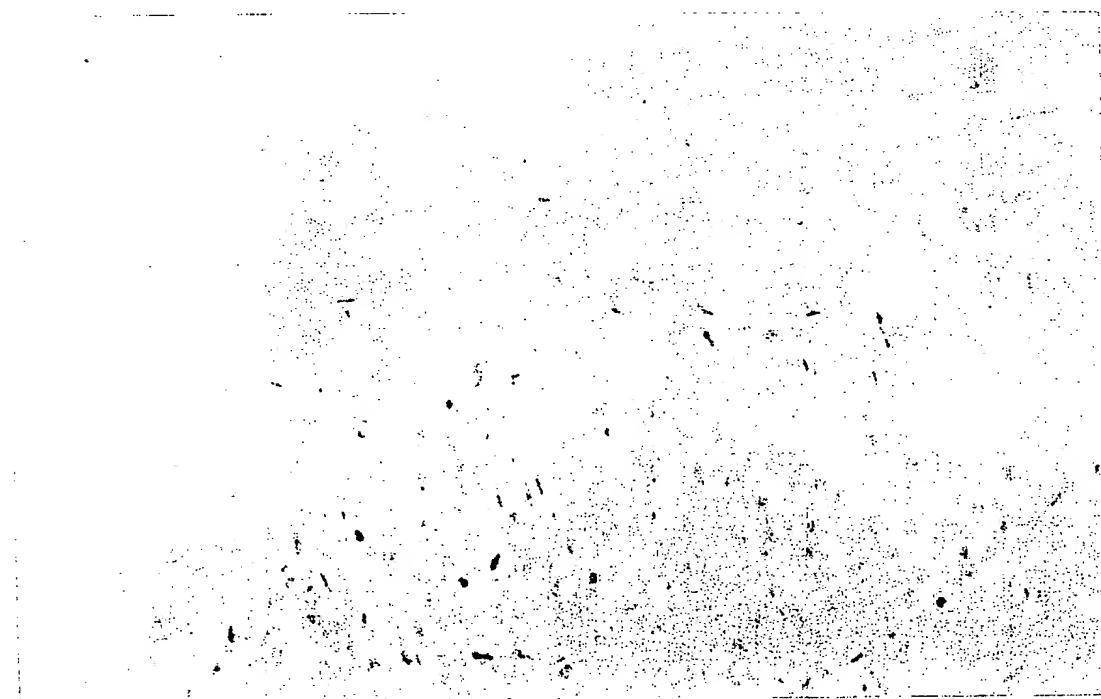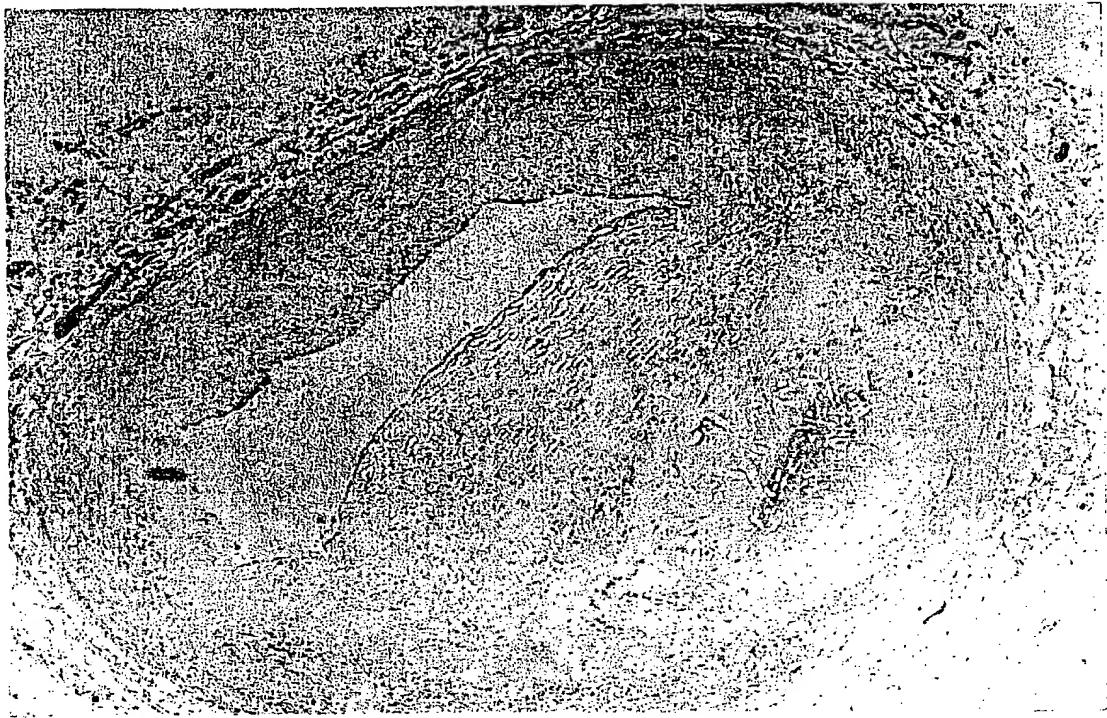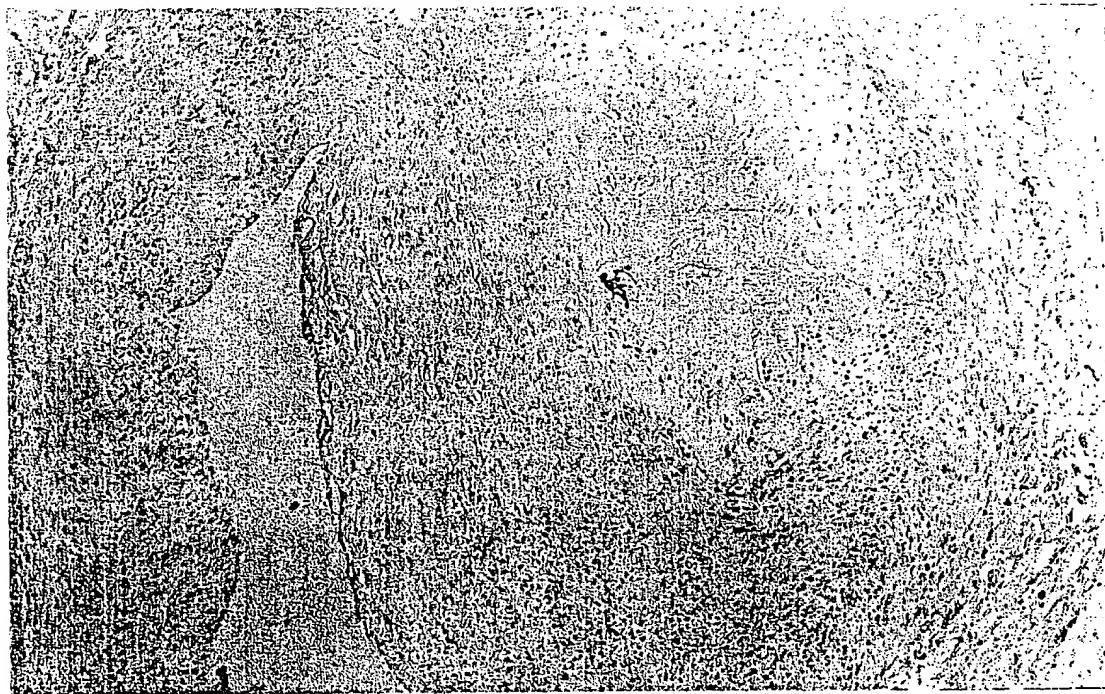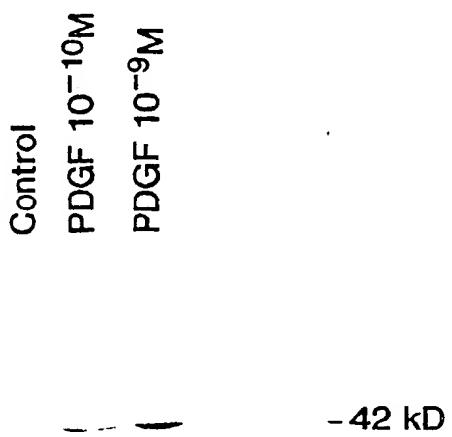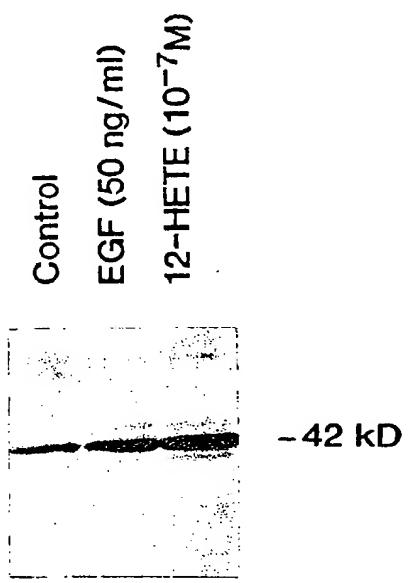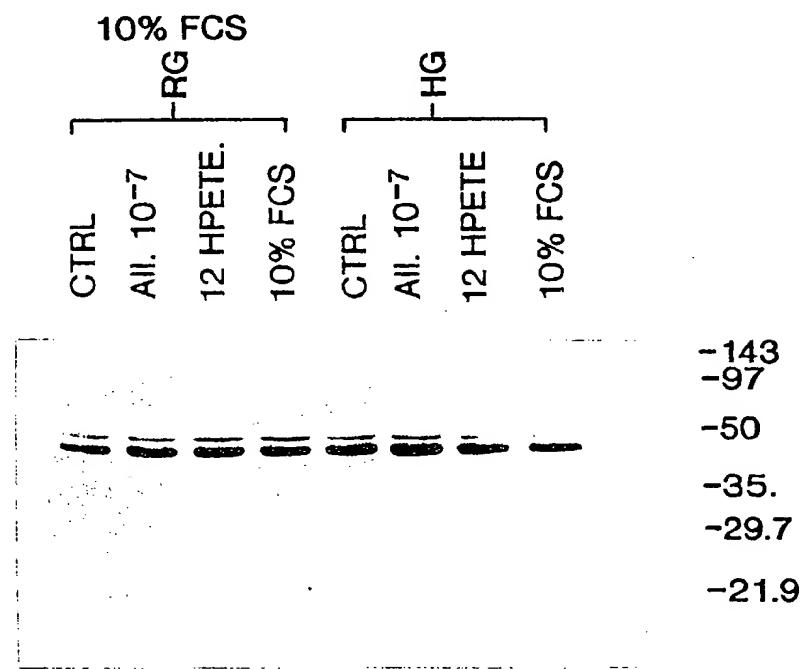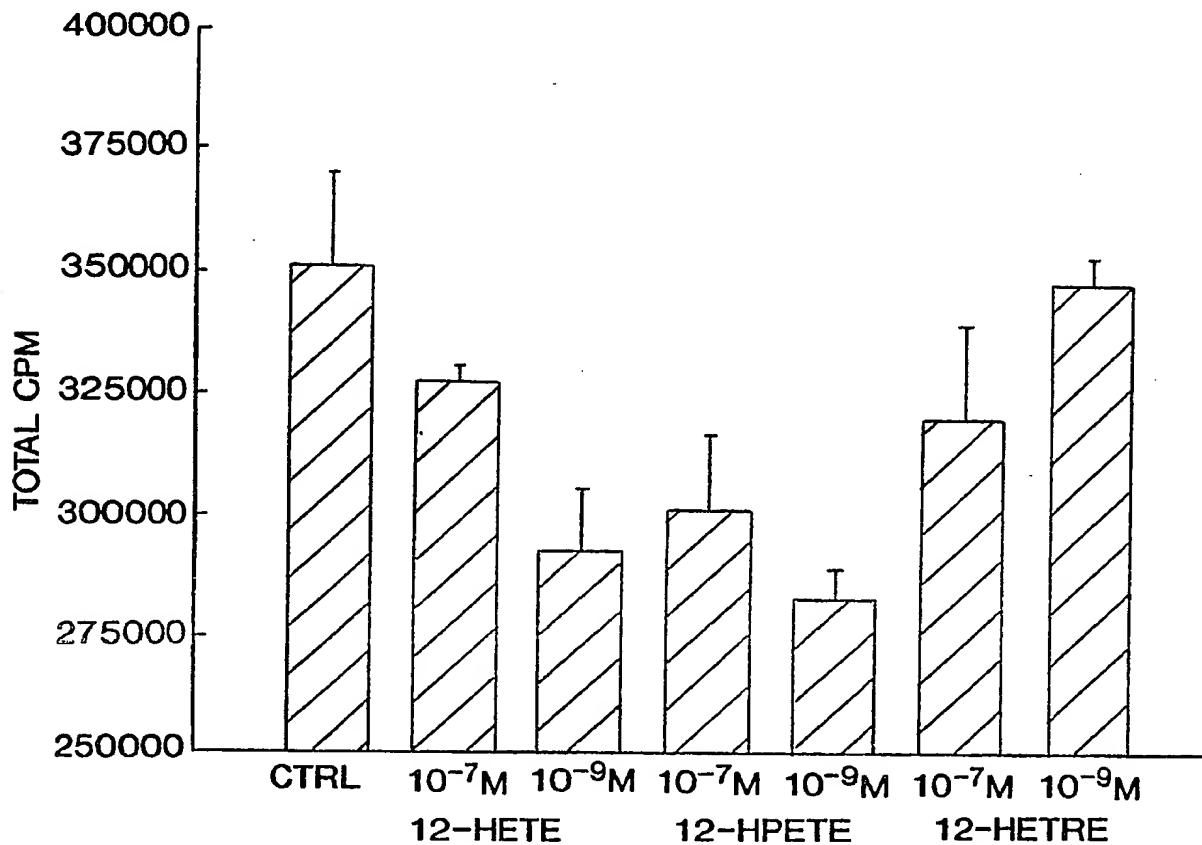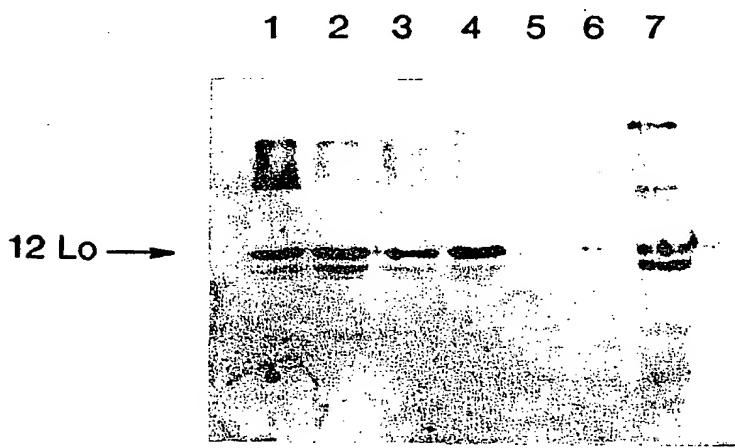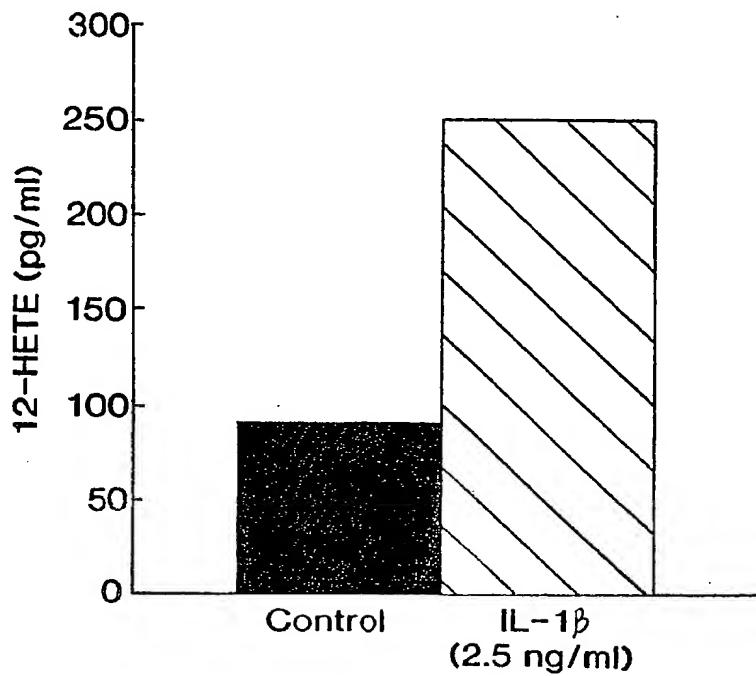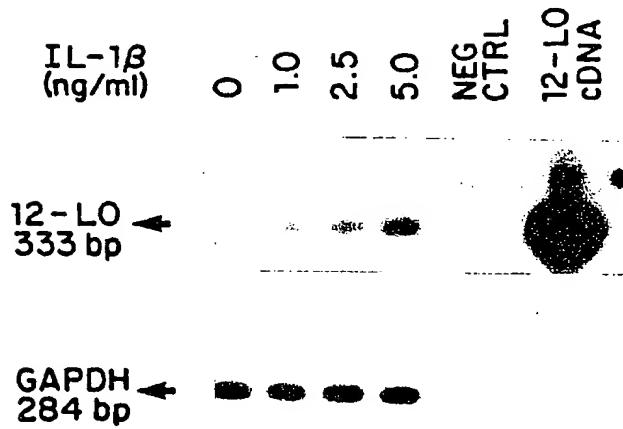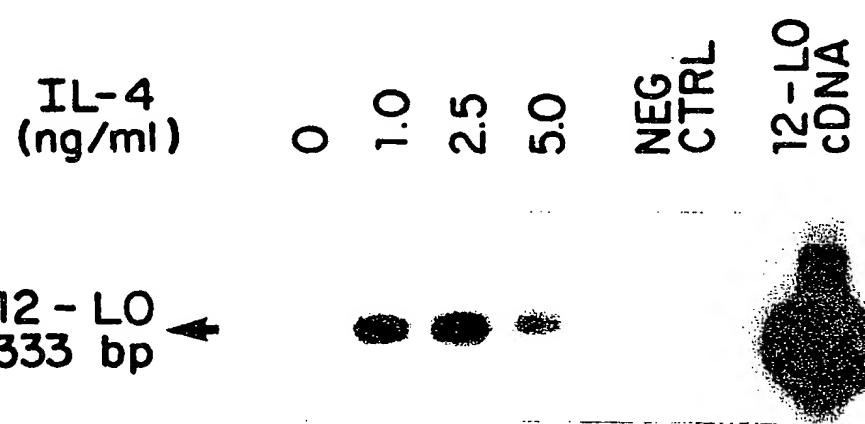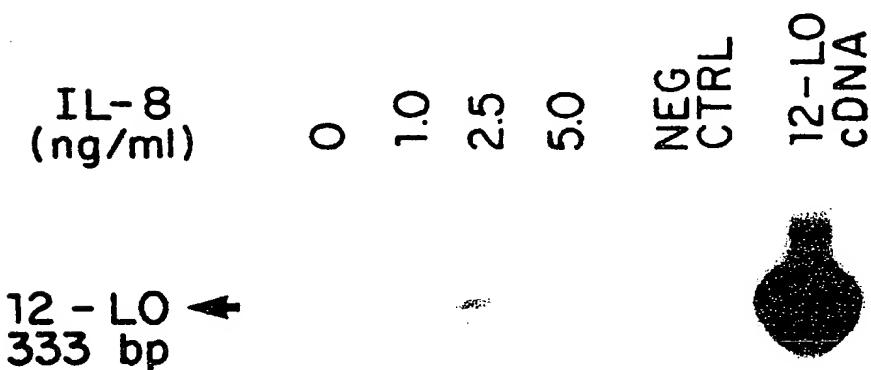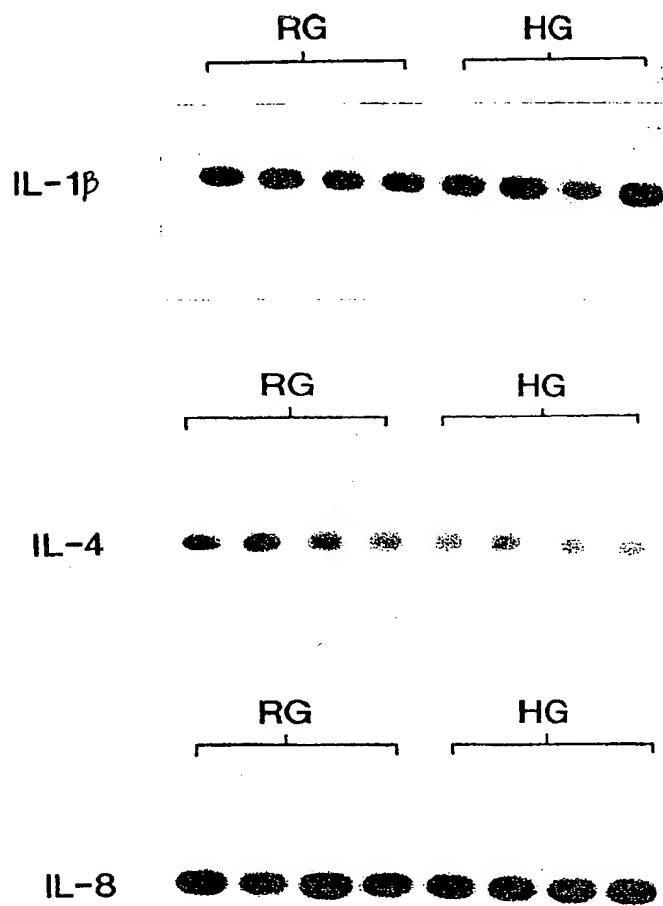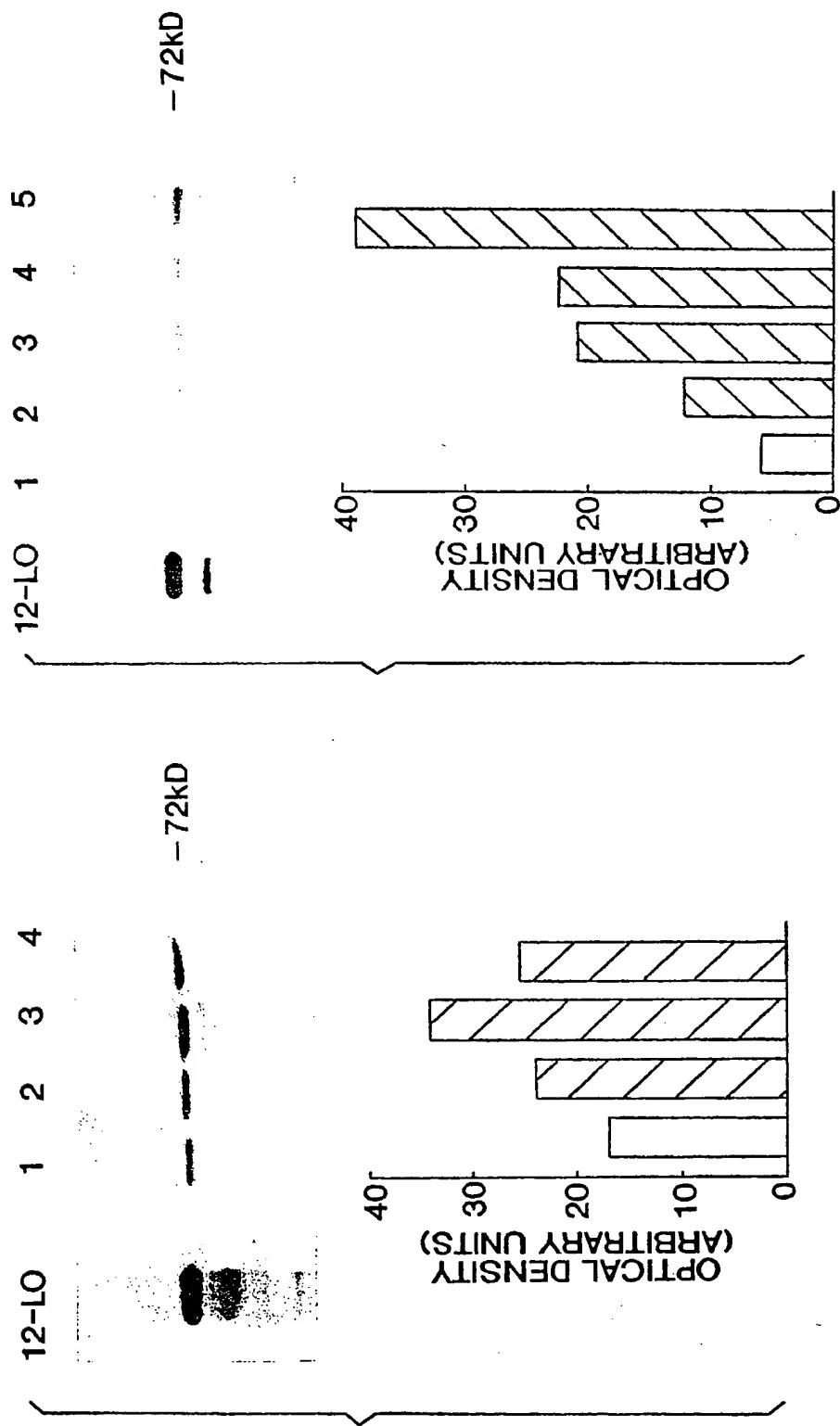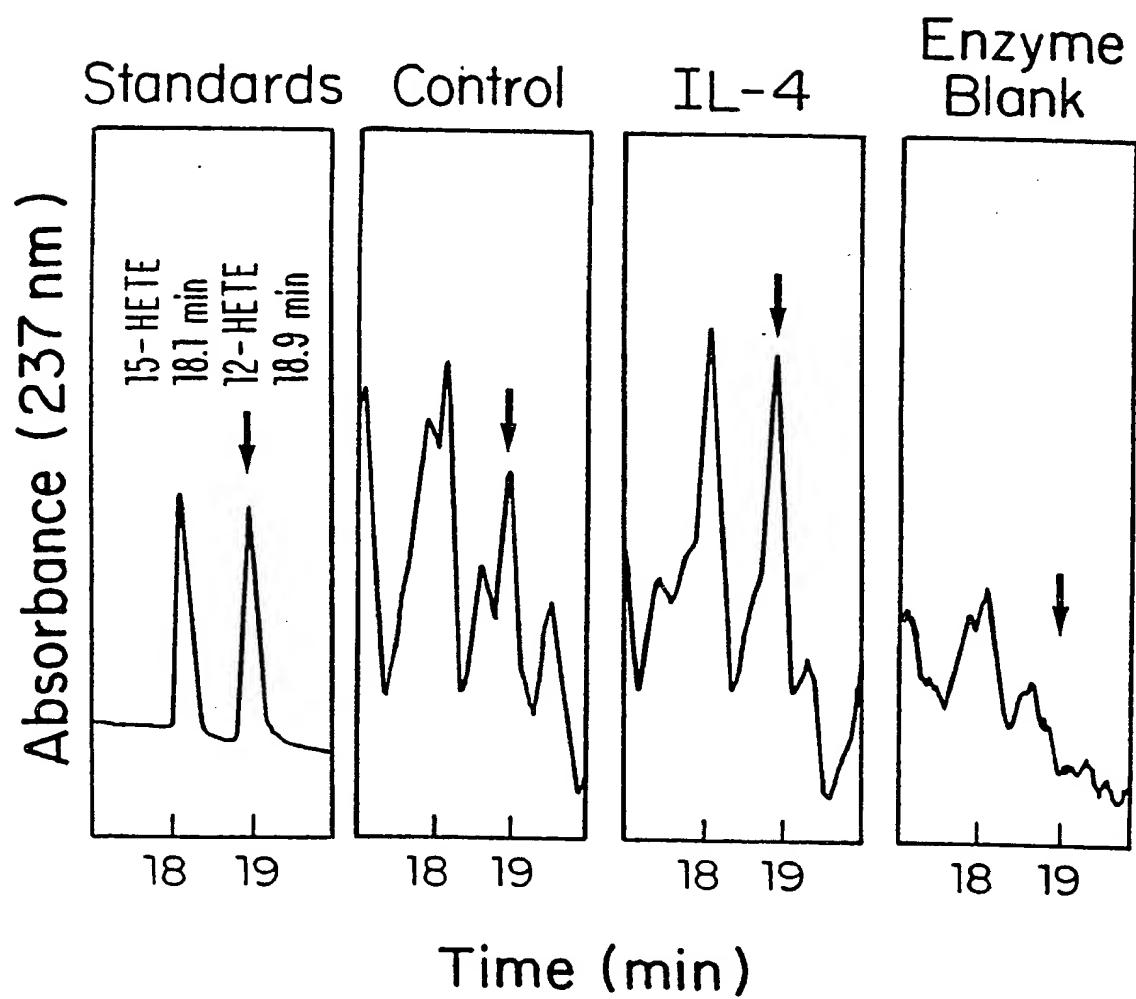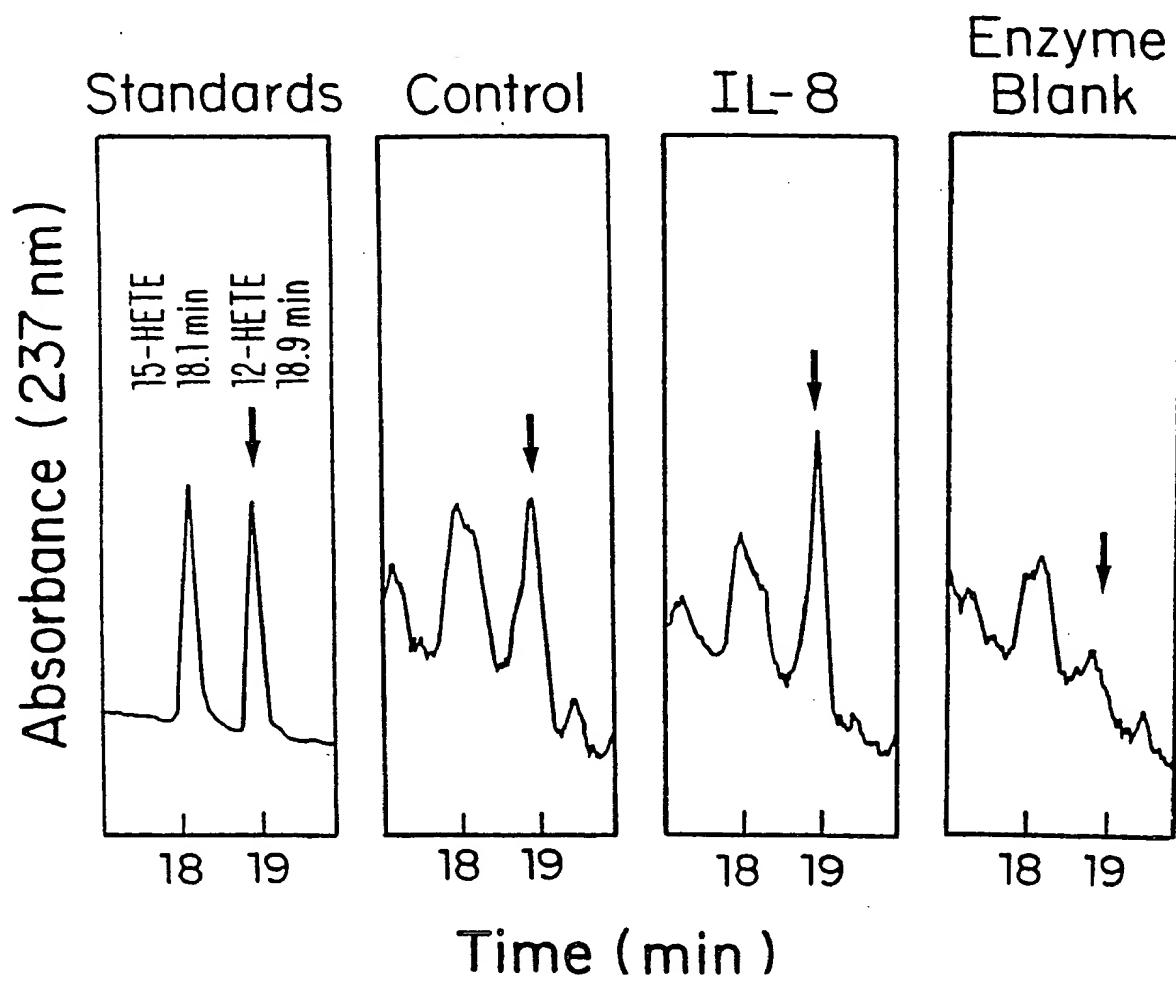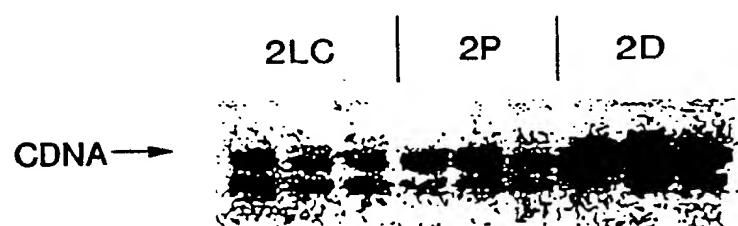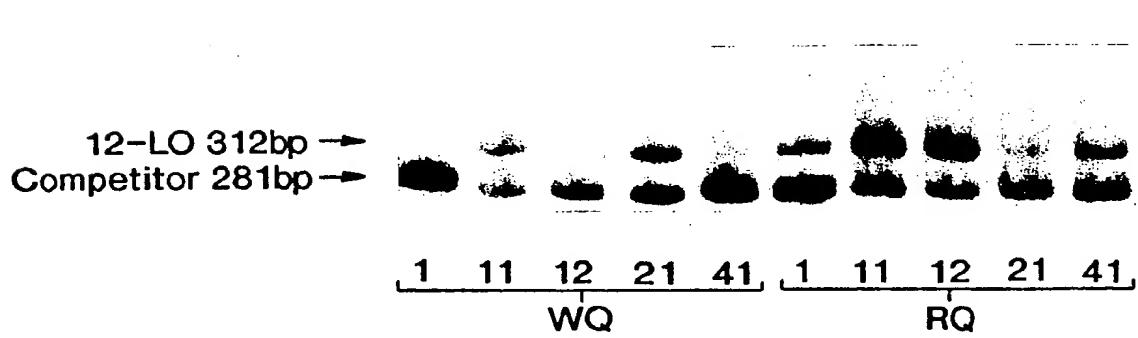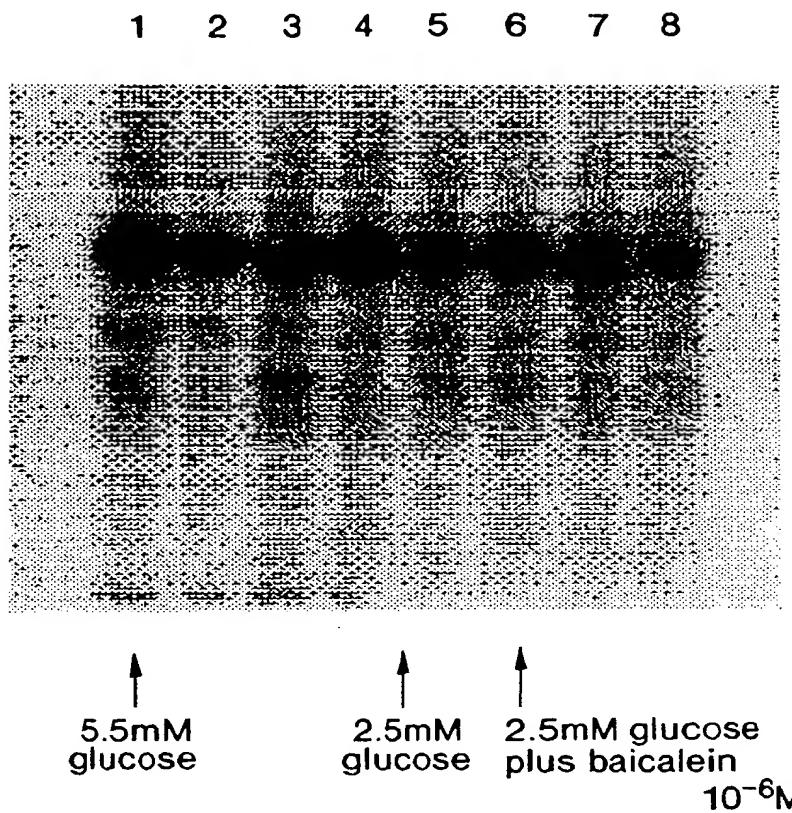
FIG. 26
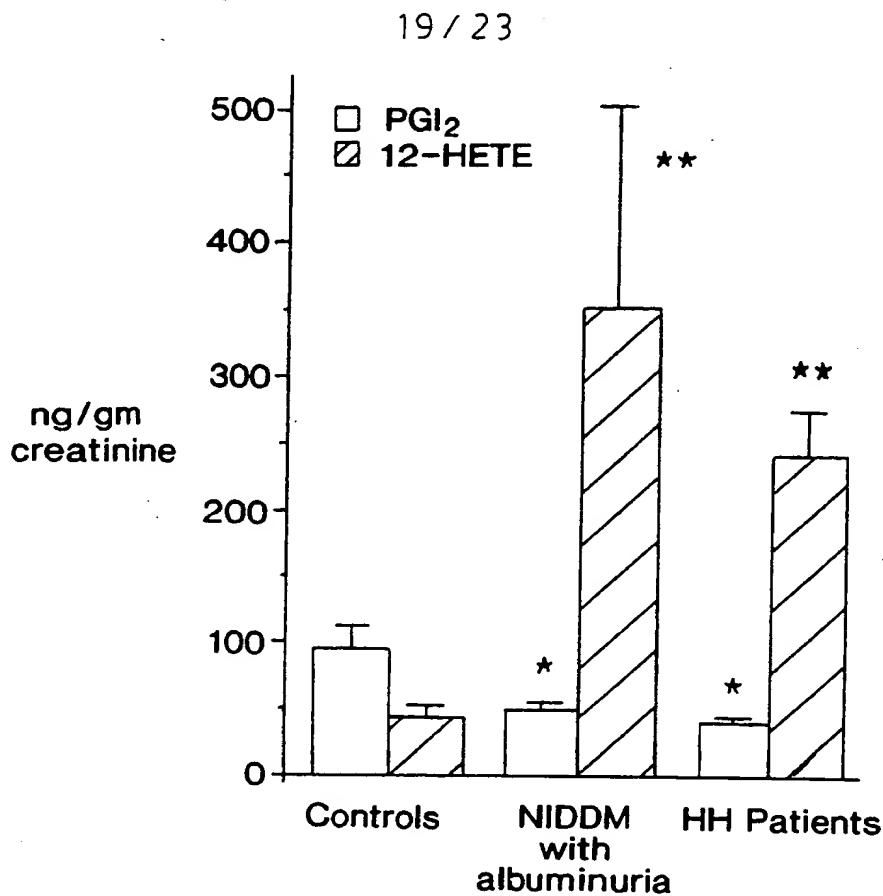
FIG. 24

FIG. 25

SUBSTITUTE SHEET (RULE 26)
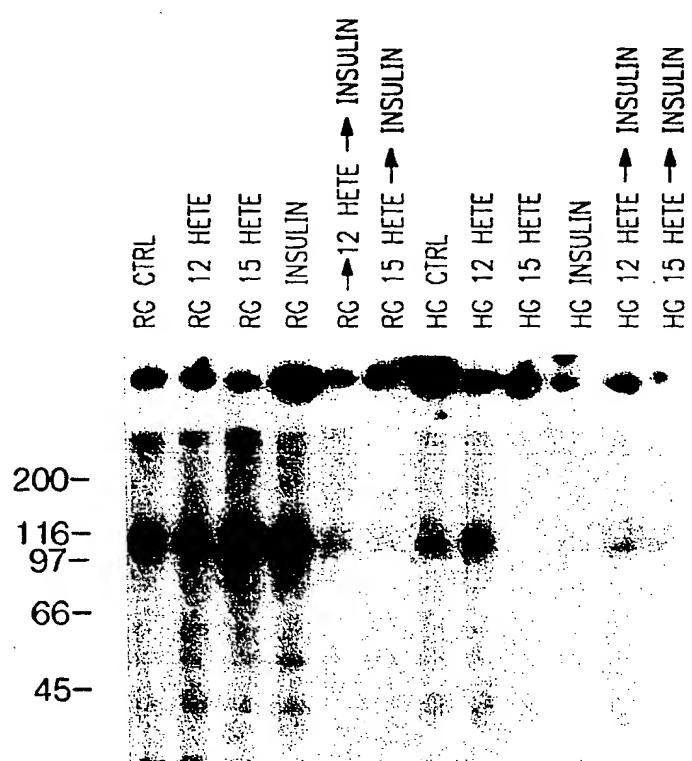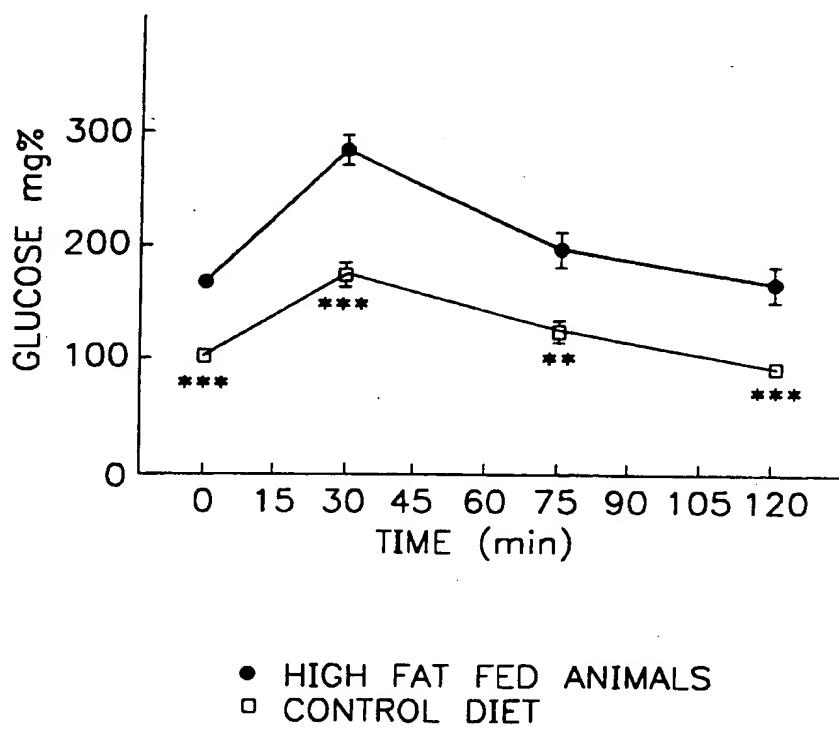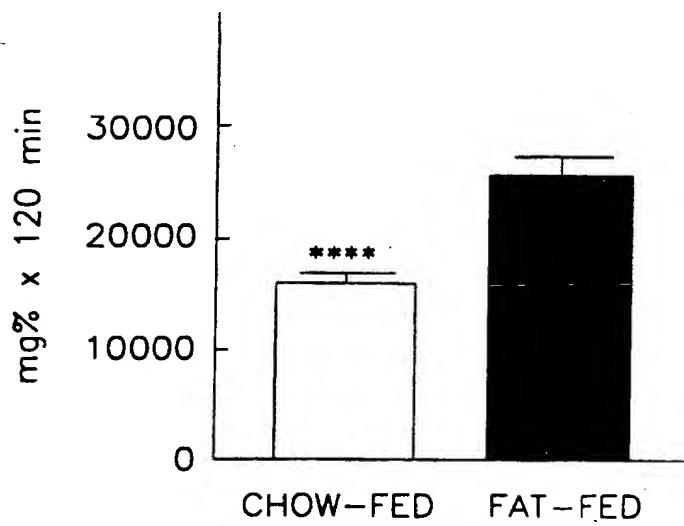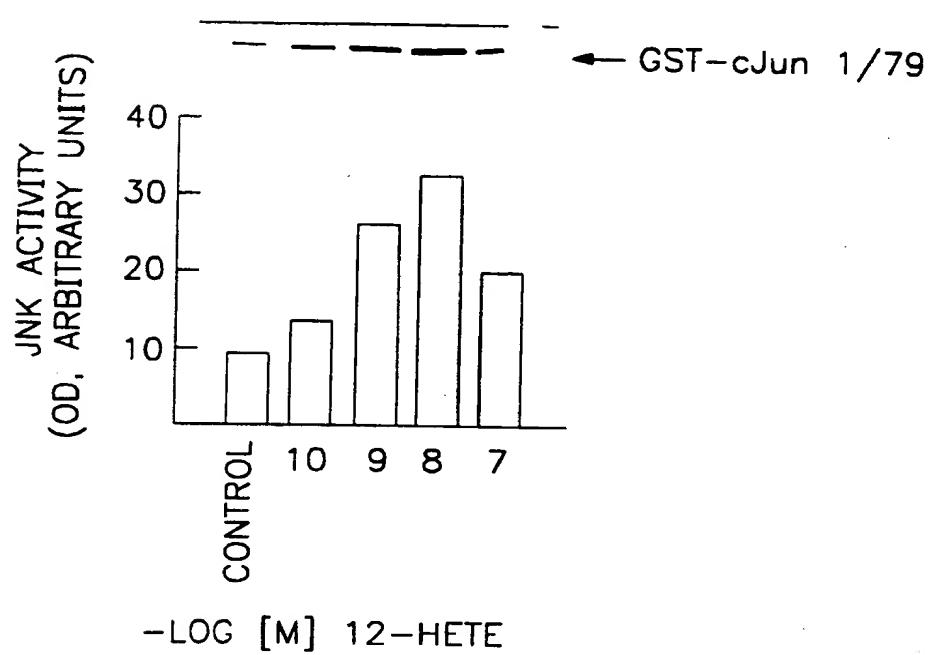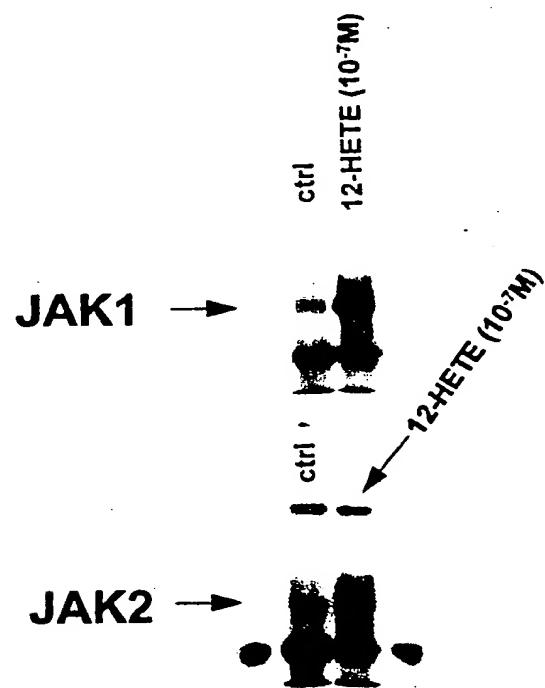
FIG. 27

● HIGH FAT FED ANIMALS
□ CONTROL DIET

FIG. 28A

FIG. 28B

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FIG. 29

FIG. 30

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N9/02   C12Q1/26   A61K31/00   A61K31/155   A61K31/35
          A61K31/44   A61K31/20   A61K38/20   A61K38/18   A61K33/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   C12Q   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 05777 (CITY OF HOPE) 17 March 1994 cited in the application see page 1, paragraph 4 - page 3, paragraph 2 see page 6, paragraph 3 - page 7, paragraph 3 see page 12, paragraph 3 - page 15, paragraph 1 see page 17, paragraph 3 - page 20, paragraph 1 --- -/--	1-11,13, 14,17, 20-37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

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'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
18 September 1996	2 7. 09. 96

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